SISTER CHROMATID EXCHANGE INDUCTION IN MAMMALIAN CELLS

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In accordance with the regulations of Edinburgh University, I declare that I have written this thesis myself and carried out all the research described except where otherwise indicated.

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GENERAL AIMS

Nearly seventy years ago Boveri (1914) suggested that somatic mutation could give rise to malignant tumours. Although it has been established that carcinogenic potency does not always correlate with mutagenic potency in mammalian systems (Miller and Miller, 1971), the relationship between oncogenic and mutagenic properties of a wide spectrum of agents warrants investigation. The effects of physical and chemical agents which are capable of inducing mutation and/or transformation in mammalian cells (and which for the sake of simplicity will be referred to as mutagens) have been studied at various levels. Mutagens have been characterized according to their cytotoxic potential, their ability to induce mutations, their capacity to induce transformation, their disruptive effects on cellular processes (DNA replication, transcription, translation, enzyme induction, etc.). By far the most studied features of mutagens are those which deal with the induction and repair of damage to cellular DNA. All known mutagens (or their metabolites) have been shown to interact with DNA. Because of this unifying characteristic a great deal of work has been invested in establishing techniques which define and/or detect mutagen-induced damage to DNA.

At the crudest level, one may detect damage inflicted on cellular DNA by observing breaks of various descriptions in the chromosome complement. Of course this method of assaying DNA-insult is limited in that it gives the observer but a small understanding of the processes which have preceded the damage. At the cytological level it is also possible to observe symmetrical and complete exchanges between sister chromatids. Again, these are empirical indices which do not of themselves further theoretical understanding of

mutagenesis and carcinogenesis.

At the biochemical level, knowledge of the quality and quantity of lesions inflicted on DNA by a given mutagen is useful for assessing the likelihood of mispairing during replication (which may or may not lead to mutation and in turn may or may not be connected with the process of transformation). Data of this sort cannot be considered informative unless they are integrated within the admittedly incomplete extant body of knowledge on DNA structure and DNA replication.

It therefore becomes apparent that no one approach to the study of mutagenesis and/or carcinogenesis can claim supremacy. In fact, each method of investigation relies on others for corroboration of its conclusions and all studies are at least tangential to the central dogma of the unity of living systems. The studies presented in this thesis concern themselves with the induction of sister chromatid exchange in two mammalian cell systems. Sister chromatid exchange (SCE) induction is a useful assay for the detection of damage inflicted on DNA by certain physical and chemical agents. Although the mechanism of SCE formation has not yet been clearly established, this assay can be used nonetheless to help answer both theoretical and applied questions. This study addresses itself to the following questions:

1. Are sister chromatid exchanges involved in the formation of chromatid aberrations? According to the exchange hypothesis (Revell, 1954)all chromatid breaks are the result of incomplete exchange. It seemed important, therefore, to investigate the coincidence of SCEs and chromatid aberrations.

2. What kinds of DNA lesions are involved in the formation of SCE? This question was approached by the use of agents for which there is some knowledge of the kinds of DNA lesions they induce. Combinations of agents were used to examine interactions of mutagens with respect to SCE induction — blocking, additivity, synergism. The agents used in this study were X-rays, ethyl and methyl methane-

sulfonate, ethyl nitrosourea and anthramycin.

3. Do inherited conditions that are associated with increased radiosensitivity as measured by induced chromosome aberration frequency also show an abnormal response to SCE induction? In this study lymphocytes from Down's syndrome individuals (which have been demonstrated to be hypersensitive to aberration induction by X-rays) were exposed to bleomycin, an agent which mimics in many ways the effects of X-rays on DNA.

4. Can the study of sister chromatid exchange be used to monitor the exposure of individuals to low levels of ionizing radiation and to detect individuals at risk of contracting hydralazine-induced systemic lupus erythematosus? In one of these studies human lymphocytes were exposed <u>in vitro</u> to low doses of gamma-radiation. In the other, lymphocytes from an individual known to be hypersensitive to hydralazine were exposed <u>in vitro</u> to this hypotensive drug.

ABSTRACT

Sister chromatid exchange (SCE) induction is regarded as an index of DNA damage. The SCE assay was used in the following studies on mammalian cells.

1. The association between bleomycin (BLM)-induced chromatid aberrations and SCE was investigated in order to evaluate Revell's exchange hypothesis for the formation of chromatid aberrations. The results of this study indicated that the exchange hypothesis can account for only some of the aberrations induced by BLM.

2. A study using chemicals which differ in their specificity for the O-6 position of guanine indicated that modification of DNA at this site is certainly not the only lesion involved in the generation of SCE. This study also included an analysis of the interactions of mutagens (blocking, additivity, synergism) with respect to SCE induction. The results are discussed in relation to DNA repair mechanisms.

3. SCE induction by a radio-mimetic agent, BLM, was investigated in lymphocytes of Down's syndrome (DS) individuals. DS lymphocytes show an enhanced response to chromosome damage by X-rays but do not differ from controls in their response to SCE induction by BLM.

4. In contrast to a suggestion in a recent report, the results obtained from an *in vitro* irradiation experiment indicated that SCE cannot be used to monitor exposure of individuals to chronic low levels of ionizing radiation.

5. It is possible that hypersensitivity to SCE induction by hydralazine (HYD) might be used to identify individuals with HYD sensitivity However, this suggestion must be regarded with caution since inter-individual differences in response to SCE induction may be wide-ranging.

ABBREVIATIONS

ANT	Anthramycin
AT	Ataxia telangiectasia
BLM	Bleomycin
BUdR	5-Bromodeoxyuridine
CHO	Chinese hamster ovary
CM	Conditioned medium
DEN	Diethylnitrosamine
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	Dimethylsulfoxide
DS	Down's syndrome
EMS	Ethyl methanesulfonate
ENU	Ethyl nitrosourea
FPG	Fluorescence plus Giemsa
G1	Pre-synthetic (DNA) phase
G2	Post-synthetic (DNA) phase
GO	Non-cycling (unstimulated) phase of lymphocytes
HYD	Hydralazine
M1	First division metaphase
M2	Second division metaphase
M3	Third division metaphase
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	Methyl nitrosourea
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
S	DNA synthesis phase
SCE	Sister chromatid exchange
SLE	Systemic lupus erythematosus
TdR	Thymidine

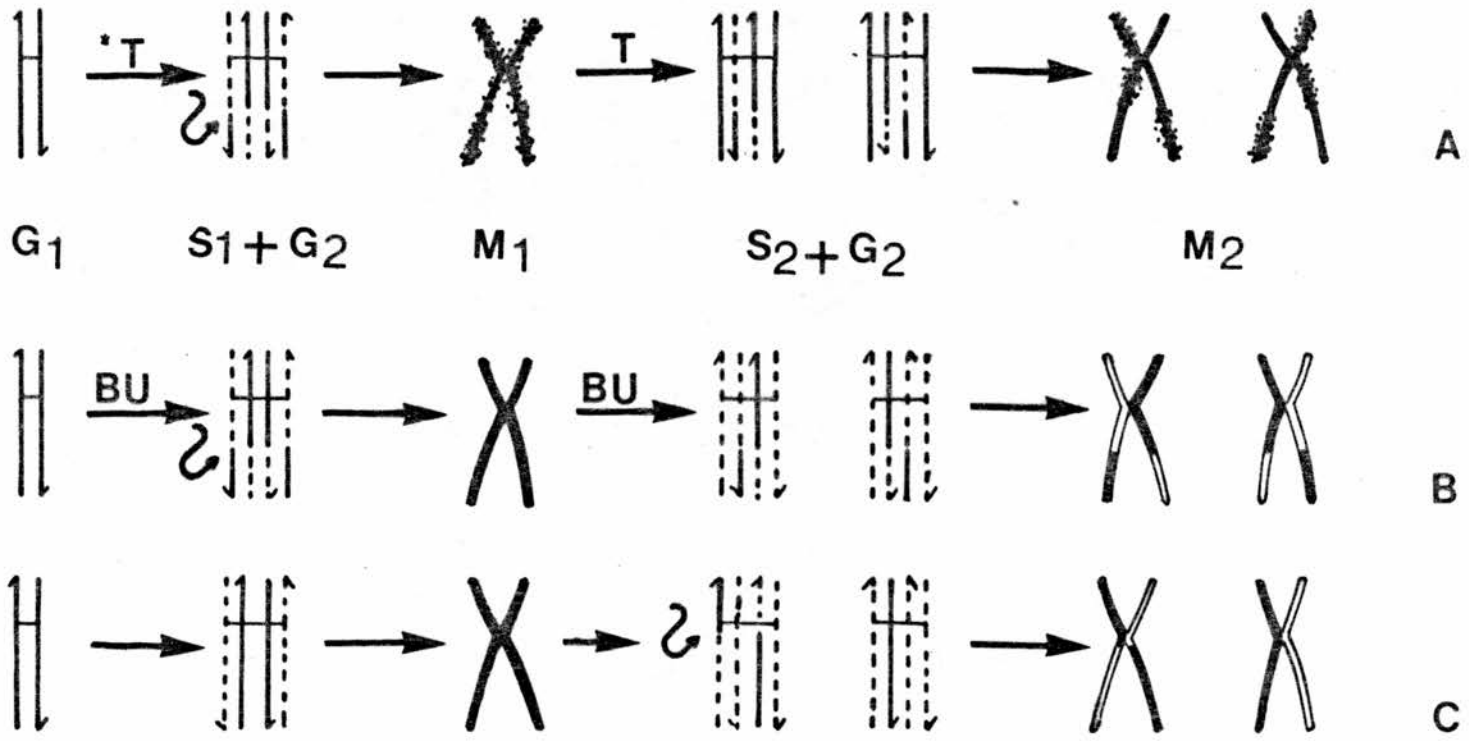
CHAPTER 1

INTRODUCTION

A. Sister chromatid exchange, a brief overview

In 1957 Taylor, Woods and Hughes described semi-conservative DNA segregation in Vicia faba cells which had been allowed to replicate once in the presence of tritiated thymidine (TdR) and then again in cold TdR. Autoradiographs of first post-labelling metaphase cells (M1) showed that both chromatids of each chromosome were equally labelled whereas in second division cells (M2) each chromosome showed one labelled and one unlabelled chromatid. These authors observed occasional label switches between sister chromatids at M2 and called these symmetrical switches "sister chromatid exchanges" (SCEs). In 1958, again using autoradiography, Taylor described semi-conservative DNA segregation in root tips of Bellavalia romana. He remarked that both subunits (DNA strands) of the chromatid DNA must be involved in an SCE since he had not observed heterolabelling (the appearance of unlabelled stretches in one of the chromatids of M1 chromosomes) which would arise from an exchange involving only one of the two subunits of DNA from each chromatid. Using the BUdR-incorporation technique for detecting SCEs (this technique will be discussed later), Wolff and Perry (1975) and Kihlman and Kronborg (1975) also failed to detect heterolabelling and reached the same conclusion as Taylor.

One important section of Taylor's study (1958) provided evidence for polarity-restricted rejoining of sister chromatid subunits. Taylor argued that tetraploid cells should exhibit a twin to single SCE ratio of 1:2 if rejoining was restricted by polarity and of less than 1:10


*T
T
A
G1
S1+G2
M1
S2+G2
M2
BU
BU
B
C

if unrestricted rejoining occurred. Twin SCEs are the result of exchanges which occur in the first cycle and are duplicated in the second cycle thereby appearing in both chromosomes of tetraploid cells (Figure 1.1). Single SCEs are SCEs that occur in the second cycle and therefore appear in only one chromosome of tetraploid cells. The results obtained by Taylor accorded with the hypothesis that polarity-restricted rejoining occurs during SCE (Taylor observed 81 twins and 30 singles). His findings were later confirmed by Wolff and Perry (1975) who analysed tetraploid Chinese hamster ovary cells and found a twin to single ratio of 1 to 2.57. These authors also analysed endoreduplicated cells and found a twin to single ratio of 1 to 2.8. Brewen and Peacock (1969a) provided evidence for polarity restricted rejoining in an autoradiographic study of dicentrics. Their method did not involve the identification of twins and singles and therefore constituted an independent evaluation of the restrictions imposed on rejoining processes. These authors analysed "mirror image" dicentrics which arose from the proximal union of isochromatid breakage induced in unifilarly tritiated TdR-substituted chromatids. Brewen and Peacock argued that two types of mirror image dicentrics could be produced:

Type-A dicentrics. Polarity restricted labelled-to-labelled and unlabelled-to-unlabelled rejoining would generate a dicentric with one completely labelled chromatid between the centromeres (Fig.1.2A).

Type-B dicentrics. Non-restricted rejoining (labelled-to unlabelled association of the subunits) would yield a dicentric with a label switch at the mid-point position between the centromeres (Fig.1.2B). Equally probable with non-restricted rejoining, is a mirror image dicentric with one completely labelled chromatid between the centromeres (this dicentric is like a type-A dicentric).

Brewen and Peacock analysed 137 mirror image dicentrics and found 104 of type-A and 6 of type-B. The remaining 27 had label switches in regions other than the mid-point

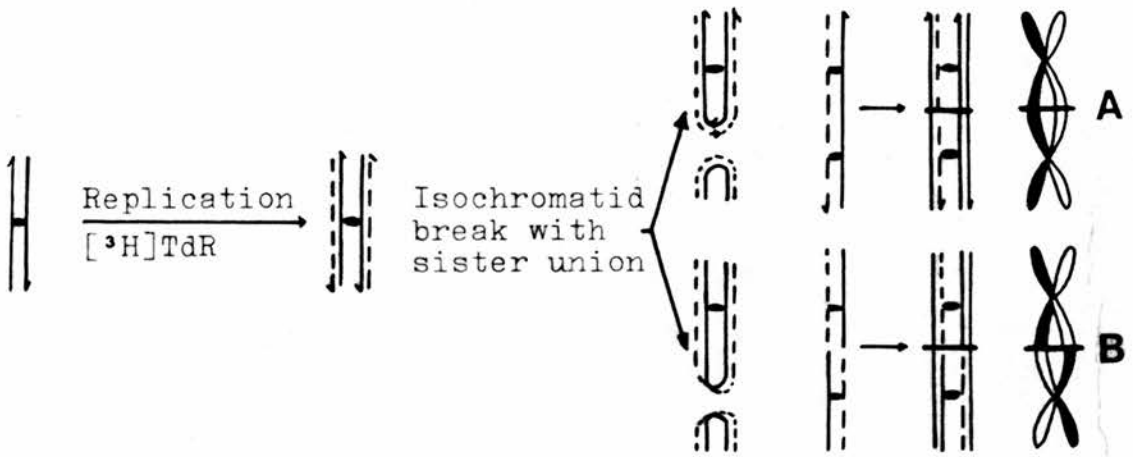


Figure 1.2. Type-A and Type-B Mirror Image Dicentrics. Type-A dicentrics arise from polarity-restricted rejoining, Type-B dicentrics arise from unrestricted rejoining. For a complete description, see the text, p. 2. Dotted lines and solid chromosome regions indicate the presence of tritiated thymidine. Figure re-drawn from Brewen and Peacock (1969a).

region (label exchange in these dicentrics had presumably occurred before or after the formation of the dicentric). Brewen and Peacock concluded that label exchange involves polarity-restricted rejoining and were able to account for the 6 type-B dicentrics by a calculation based on observed exchange frequency per micron of chromosome length and autoradiographic resolution.

Early experiments investigating spontaneous and induced SCEs were hampered by the poor resolution afforded by the autoradiographic technique. One notable point of contention was that of isolabelling. Isolabelling consists of an autoradiographic image over homologous regions of sister chromatids in M2 chromosomes where only one chromatid should be labelled at any one locus after incorporation of [^{3}H] TdR in the first cell cycle. The notion that isolabelling was caused by anything other than the inability to resolve very closely adjacent SCEs was dispelled by a study of autoradiographic image spread (Gibson and Prescott, 1973) in which the track length of beta-particles was measured and found to be approximately 1 micron. Most isolabelled regions measured by Gibson and Prescott were between 1 and 2 micron in length (the estimated image overlap for a single SCE). Isolabelled regions larger than 2 microns in length were few and could be accounted for by multiple SCEs in these regions. Isolabelling has not been observed since the development of more sophisticated methods for sister chromatid differentiation (Wolff and Perry, 1974, Korenberg and Freedlender, 1974).

The development of high resolution sister chromatid differentiation techniques began with the observation that treatment with the thymidine analogue 5-bromodeoxyuridine (BUdR) caused reduced staining intensity of the late-replicating X chromosome of embryonic rat cells (Huang, 1967). A battery of techniques with increasing resolution capacity were developed on the assumption that BUdR caused despiralization of DNA strands into which it had been incorporated (Zakharov and Egolina, 1972, 1974, Latt, 1973). Latt (1973) described a difference in staining intensity between uni-

and bifilarly substituted chromatids stained with the fluorochrome Hoechst 33258. This discovery led to the publication of no fewer than three independently developed techniques for permanent "harlequin" staining (Perry and Wolff, 1974, Korenberg and Freedlender, 1974, Kim, 1974). The most widely adopted method for producing differentially stained sister chromatids, the fluorescence plus Giemsa (FPG) method, was developed by Perry and Wolff (1974) who used visible light to quench the fluorescence of the Hoechst 33258 with which they had treated their slides. Light treatment of the slides was followed by hot salt treatment and finally by Giemsa staining. This procedure produces permanent preparations which do not require immediate analysis under a UV microscope as did those of Latt (1973) because of rapid fading of fluorescent preparations. Examples of "harlequin" stained chromosomes are presented in Figures 1.3. to 1.5. and a schematic description of SCE detection by the BUdR-incorporation method is included in Figure 1.1.

B. Spontaneous sister chromatid exchanges

Both tritiated thymidine (Gibson and Prescott, 1972) and BUdR (Kato,1974a, Tice et al., 1976, Wolff and Perry, 1974) can induce SCEs. Consequently the question of whether SCE is ever a spontaneous event has been raised. This, however, is a rhetorical question since, in the absence of known and experimentally controlled mutagens, cellular metabolites are quite likely to induce SCEs (remark made by Professor Evans in the course of discussing this point).

Circumstantial evidence exists for the occurrence of SCE in the absence of known mutagens. If an exchange occurs between the sister chromatids of a ring chromosome, the chromatids fail to separate at anaphase and a double-sized ring can be observed in the succeeding metaphase. Double-sized rings have been observed in mitotic maize cells

Figure 1.3. Harlequin Stained Metaphase Chromosomes. Sister chromatid differentiation has been achieved by a modification of the fluorescence plus Giemsa method in this second division lymphocyte from a normal male.

Figure 1.4. SCEs in a Human Lymphocyte Exposed to Mitomycin C
Lymphocytes from Control 2 (Chapter 5) were exposed to
1 x 10^{-7}M mitomycin C for 72 hours.

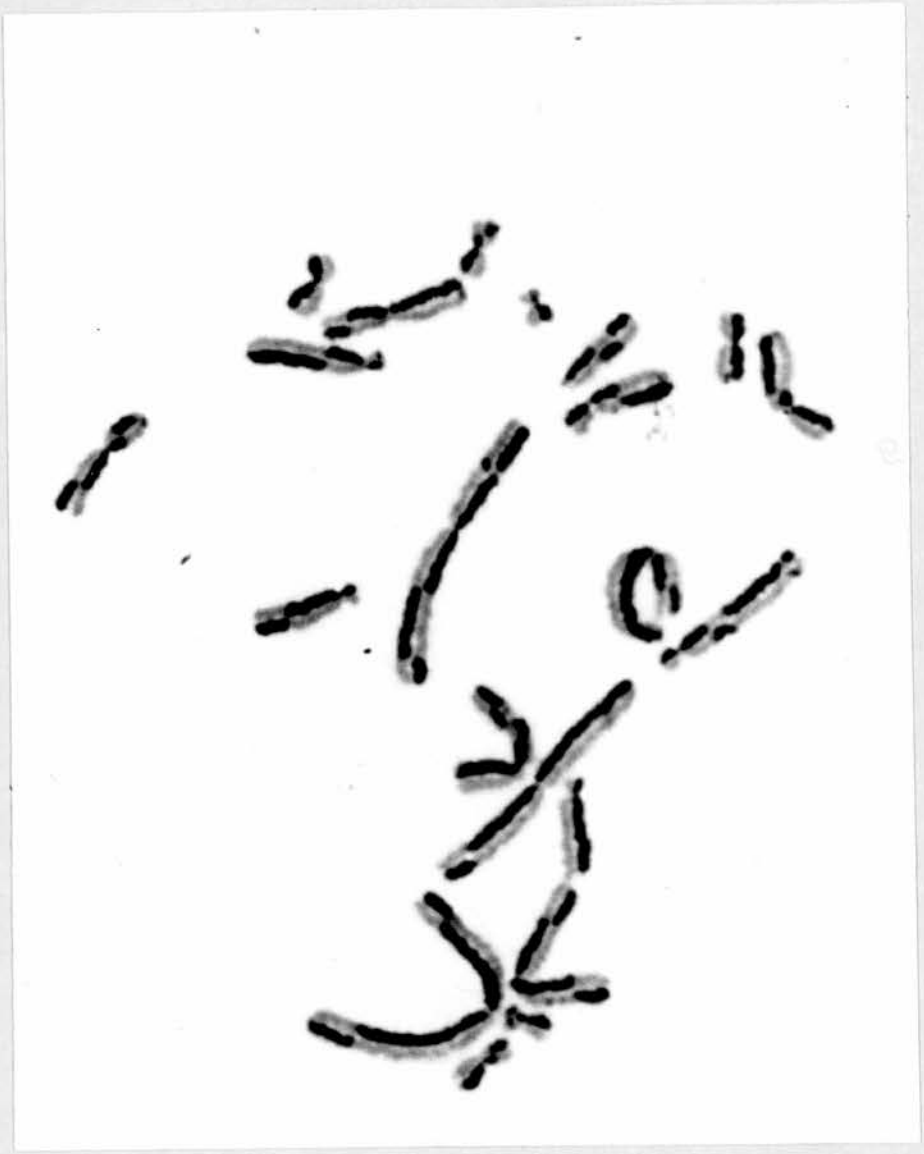


Figure 1.5. Ethyl Methanesulfonate Induced SCEs. Chinese hamster ovary cells were exposed for 24 hours to 5×10^{-4} M ethyl methanesulfonate. This second division metaphase shows differentially stained chromosomes with 32 SCEs.

in the absence of mutagen (McClintock , 1938). In meiotic maize cells, Scwhartz (1953) analysed anaphase configurations resulting from crossing over between a large dicentric ring and its homologous rod chromosome. Schwartz reported a large discrepancy between the observed and expected anaphase configurations and concluded that this discrepancy could be accounted for by sister chromatid crossing over. Brewen and Peacock (1969b) studied the behaviour of a ring chromosome in successive anaphases of human peripheral blood lymphocytes. From their study these authors concluded that although tritiated thymidine increased the frequency of dicentric ring formation, the induced SCE frequency was tritium dose independent because comparable levels of dicentric rings were induced in the first and second cycle (the amount of tritium in the second cycle is half of that of the first cycle). From these findings they concluded that a certain proportion of SCEs were spontaneous events.

The now well-established notion that a certain proportion of SCEs reflect spontaneous sister chromatid exchange processes does not however explain inter- and intraspecific differences in base-line SCE frequencies. Base-line SCE frequencies vary from less than one SCE per cell in Drosophila (Gatti et al., 1979) to nearly one hundred SCEs per cell (lymphocytes) of individuals with Bloom's syndrome (Chaganti et al., 1974). Of course, these are extreme cases and most base-line SCE frequencies cluster around ten SCEs per cell. Kato (1977a) studied base-line SCE frequencies in primary lung cultures of 23 species of mammals and found a positive correlation between genome size and SCE frequency. Therefore, interspecific variability of base-line SCE frequencies can be partly accounted for by differences in DNA content and chromosome length between species (Drosophila has only 1/20th the amount of DNA in a mammalian cell, Wolff, 1977). Observed intraspecific differences in SCE frequencies in man cannot be attributed to interindividual differences with respect to age, sex (Galloway and Evans, 1975) and karyotypic abnormalities (Stoll et al.,

1977, Lezana et al., 1977) and are therefore thought to reflect more subtle interindividual differences which at present are undefined. It must be pointed out that it is often difficult to draw comparisons between base-line SCE frequencies obtained by different laboratories because of variation in the protocols used. BUdR concentrations, for example, vary widely between research groups (Kato, 1977a).

In man, SCE distribution within the chromosome complement appears to be related to the lengths of the chromosomes in which the SCEs occur. However, several authors (Galloway and Evans, 1975, Latt, 1974a, Chaganti et al., 1974) have reported an under-representation of SCEs in the small chromosomes of the human karyotype (E, F and G groups). On the other hand, the longest chromosome of Chinese hamster ovary cells usually bears more SCEs than expected if SCE distribution in the chromosome complement varies with length alone (Ikushima and Wolff, 1974).

Differences in SCE frequency between centromeric, heterochromatic and euchromatic regions have been reported but the findings are conflicting. For instance, Galloway and Evans (1975) reported an excess of exchanges in the mid-arm regions of human chromosomes whereas Tice et al. (1975) reported the opposite. Increased incidence of SCEs has been observed in heterochromatic regions of meadow vole chromosomes (Natarajan and Klášterská, 1975). However, decreased frequency of SCE in heterochromatic regions has been observed in montane vole chromosomes (Hsu and Pathak, 1976). Carrano and Wolff (1975) reported that, in Indian muntjac chromosomes, SCEs expected to be in the C band regions occurred almost exclusively at the junctions between C and non-C blocks. Bostock and Christie (1976) reported the same for kangaroo rat. Finally, neither over- nor under-representation of SCEs was observed in the heterochromatic long arms of the X chromosome of Chinese hamster (Kato, 1974d). While it should be recognized that these studies were performed on different organisms, it should be conceded that the picture is too confused for one to be able to make a general statement with respect to

the distribution of base-line SCEs within a chromosome complement.

C. Induced SCEs

(i) BUdR induced SCEs

Although the thymidine analogue BUdR is used to detect the induction of SCEs, there is evidence that the analogue itself induces SCEs. The study of BUdR capacity to induce SCEs is therefore of crucial importance. BUdR induction of chromosomal aberrations is a well-established phenomenon (Hsu and Somers, 1961, Dewey and Humphrey, 1965, Huang, 1967). As soon as BUdR-labelling techniques for sister chromatid differentiation got underway, the effects of BUdR concentration on SCE induction were investigated. Wolff and Perry (1974), Latt (1974b) and Kato (1974a) published results which clearly demonstrated that SCE frequencies rise with increasing BUdR concentration but plateau at fairly low concentrations of the analogue. There is now good evidence in support of the notion that base-line SCEs are dependent on the amount of BUdR available per cell and not to the amount of BUdR incorporated into cellular DNA Davidson et al., 1980, Stetka and Carrano, 1977). Therefore BUdR concentrations are best kept fairly low (but of course high enough to achieve good differential staining) and at the same time within the plateau range. The analogue concentrations used in this study were selected to meet both these citeria.

An indirect consequence of BUdR substitution on increasing SCE levels is that of radiosensitization. Radiosensitizing properties of this thymidine analogue were investigated long before its properties as a sister chromatid differentiating agent were known (Djordjevic and Szybalski, 1960). Incorporation of BUdR into DNA was observed to result in decreased cell survival following irradiation with X-rays (Djordjevic and Szybalski, 1960). BUdR-substi-

tuted DNA exhibits increased sensitivity to break induction by X-rays (Somers and Humphrey, 1963), gamma rays (Dewey and Humphrey, 1965), UV light (Erikson and Szybalski, 1961 and 1963) and visible light (Ben-Hur and Elkind, 1972). Later these properties were used to investigate mechanisms of chromosome breakage (Bender et al., 1973b, 1974, Natarajan et al., 1980b). Grain count measurements of the degree of BUdR incorporation into DNA indicate that sensitization to gamma irradiation does not occur until at least 25% substitution has been achieved (Dewey and Humphrey, 1965). Recently Wolff and Fijtman (1981) performed a study of DNA X-ray sensitization by BUdR. They concluded that the degree of substitution is a critical factor in determining sensitivity to X-ray induced breakage. Wolff and Fijtman found that when over 60% of the DNA is substituted three times as many breaks are produced in BUdR-containing DNA as in native DNA. It now seems quite likely that BUdR sensitization of DNA is responsible for at least some of the increase in the incidence of SCE following exposure to ionizing radiation (see Chapter 6 for a more comprehensive discussion), UV (Kihlman et al., 1977) and visible light (Kato, 1977b and 1974b).

(ii) Ultraviolet light and ionizing radiation-induced SCEs

If cells are irradiated with UV and then allowed to replicate twice in the presence of BUdR, a UV dose dependent increase in the frequency of sister chromatid exchange can be observed in second division cells (Kato, 1973, Wolff et al., 1974). In this case, UV-induced SCEs are independent of BUdR radiosensitizing effects since cells are irradiated prior to incorporation of the analogue. The induction of SCEs by UV is an S-dependent process: that is cells must undergo DNA replication before exchanges can be detected (Kato, 1973, Wolff et al., 1974). Capacity to excise UV induced dimers does not appear to be directly correlated with SCE frequency. Although UV irradiation of xeroderma pigmentosum cells (which have an inefficient UV-

endonuclease, Mortelmans et al., 1976) induces more SCEs than in similarly irradiated control cells, it is not clear that the increase in SCE can be closely correlated with the degree of excision repair in xeroderma cells (de Weerd-Kastelein et al., 1977, Wolff et al., 1977, Cheng et al., 1978). Wolff et al. (1974) also reported no correlation between the degree of dimer excision repair capacity of rodent cell lines and the frequency of UV induced SCEs. A dose of UV which induces approximately 20,000 dimers induces only one SCE (Reynolds et al., 1979). Consequently one can appreciate that the dimer excision repair system is not likely to be the key repair system contributing to our understanding of the process of SCE induction. The SCE-initiating lesions induced by UV light still remain uncharacterized.

Ionizing radiation-induced SCEs are few, if there are any. This may not be surprising since ionizing radiation induced base damage is repaired by short-patch repair involving only 3-4 nucleotides (Regan and Setlow, 1974). An entire chapter of this thesis addresses itself to the question of SCE induction by ionizing radiation. In order to avoid repetition the reader is referred to Chapter 6.

(iii) Chemical mutagen induced SCEs

A bewildering variety of chemicals induce SCEs. It is not within the scope of this study to present a comprehensive review of the effects of the myriad chemicals which induce SCEs. The reader is referred to a recent review written by Perry (1980) in which some one hundred of these chemicals are itemized. Briefly, compounds which induce SCEs can be divided into two main categories: direct-acting compounds and indirect-acting compounds. Direct acting mutagens, notably alkylating agents (e.g. EMS, MMS, MMC)[1] possess functional groups which are capable of acting im-

1. See Abbreviations List

mediately on DNA. It has been inferred that some of the DNA lesions they produce are responsible for initiating SCE. Some direct-acting mutagens are also crosslinking agents (MMC, 8-methoxypsoralen plus near UV light, nitrogen mustard, Myleran).

Indirect-acting agents acquire DNA-attacking functional groups only after metabolic activation. Polycyclic aromatic hydrocarbons and aromatic amines are included in this category and are important groups of mutagens. Indirect-acting mutagen SCE induction is rather more difficult to achieve than SCE induction by direct-acting mutagens. While the latter can be achieved in any cell test system, the former requires that cells be capable of metabolizing the promutagen to its DNA-damaging derivative(s). Most fibroblast cell lines lack this capacity. The problem may be circumvented by using cells which have retained their ability to activate promutagens (e.g. lymphocytes, primary fetal cells, RL cells described by Meyer and Dean, 1981). Alternatively, in vitro activation may be achieved by using S9 mix, an S9 microsomal fraction first described by Garner (1973) and an NADPH generating system (Stetka and Wolff, 1976, Natarajan et al., 1976) to metabolize extracellularly the promutagen to its active form. Cells that would otherwise not exhibit an increase in SCE frequency after treatment with indirect-acting agents (e.g. Chinese hamster ovary cells) exhibit increases in SCE frequency if incubated in the presence of the promutagen and S9 mix (Takehisa and Wolff, 1977, Stetka and Wolff, 1976b). However, because the microsomal mixed function oxidase system can be primarily regarded as a metabolizing and detoxifying system (De Flora, 1978), it is not surprising that S9 mix also has the ability to deactivate certain mutagens. This can be inferred from the findings of Wolff and Takehisa (1977). These authors were unable to induce SCEs in Chinese hamster cells following treatment with aminofluorene and acetylaminofluorene in the presence of S9 mix but obtained large increases in the SCE frequencies of Chinese hamster cells exposed to the active derivatives of these compounds in the

absence of S9 mix.

Finally, both indirect and direct-acting mutagens have been demonstrated to induce SCEs in vivo: in experimental animals (Allen and Latt, 1976a, Allen et al., 1977, Bayer and Bauknecht, 1977, Vogel and Bauknecht , 1976, Schreck et al., 1979) and in humans treated with cytostatic drugs (Perry and Evans, 1975, Nevstad, 1978, Raposa, 1978). It is interesting and important to note that DNA replication after damage (be it by direct- or indirect-acting mutagens) is required for the expression of SCEs at the ensuing metaphase.

D. Formation of SCEs

It should be by now apparent that, given the heterogeneity of SCE inducing agents, drawing up a general model for the process of SCE formation is a difficult task. To date, the events which cause initiation and manifestation of SCEs have not been unequivocally described. However, several models have been advanced to account for the formation of SCEs. These models must take into account the forementioned observations that:

1. SCE is a polarity-restricted double strand exchange event
2. that X-rays are inefficient at inducing SCE
3. that UV and most chemical mutagens in their active forms induce SCEs
4. that post-damage DNA replication is required for the expression of SCEs
5. that a wide variety of DNA lesions are produced by SCE-inducing agents.

The replication bypass model for SCE proposed by Shafer (1977) invokes a mechanism whereby DNA crosslinks are bypassed without being repaired and persist despite SCE formation. Severe critcism of this model has been advanced by Stetka (1979) who pointed out that Shafer's model is inadequate because, among other things, it cannot account for the efficient induction of SCEs by non-crosslinking agents.

Recombinational repair models based on Whitehouse (1963) and Holliday (1964) have been proposed to account for the generation of SCEs (Figure 1.6). In bacteria, repair of lesions during the post-replication or S-phase follows the formation of gaps remaining in the daughter DNA strand opposite the original lesions in the parental strand. These are later filled in by recombinational repair with the complementary parental DNA strand (Rupp et al., 1971). However, Lehmann (1972) has questioned the existence of such a process in mammalian cells because his data suggest that gap-filling occurs by de novo synthesis in mammalian cells and not by a recombinational repair process. Hybrid DNA (expected to be formed by recombinational repair) has been detected in Chinese hamster ovary cells (Rommelaere and Miller-Faurès, 1975, Higgins et al., 1976, Moore and Holliday, 1976). Moore and Holliday pointed out that the amount of hybrid DNA found in their experiment was 17 to 25 times in excess of the amount of hybrid DNA which might be necessary for the formation of SCEs. Therefore, they could not rule out that hybrid DNA in their experiment had been formed by some other mechanism as well as by recombinational repair. Loveday and Latt (1978) presented evidence against Moore and Holliday's proposal that isolation of hybrid DNA in Chinese hamster cells could be taken as an indication that SCE arises via recombinational repair. Loveday and Latt isolated a small amount of unusually dense double stranded DNA from Chinese hamster ovary cells grown in BUdR. The amount of this DNA was found to be 100 times in excess of that expected based on the SCE frequency of these cells. The amount of dense double stranded DNA could neither be decreased by growing cells for one more cell cycle in the presence of thymidine nor increased by treatment with mitomycin C. Furthermore, dense double stranded DNA was isolated whether or not BUdR had been incorporated into DNA. Loveday and Latt concluded that the DNA they had isolated and which had previously been interpreted as being bifilarly substituted with BUdR (by Moore and Holliday, 1976, and Rommelaere and Miller-Faurès

1976) could not be presented as evidence for the recombinational repair model for SCE but rather that it might merely be Chinese hamster ovary cell satellite DNA.

Several studies have provided evidence for the intimate association between SCE formation and DNA replication. Exposure to 8-methoxypsoralen plus near UV induces SCEs which are restricted to those regions which are unreplicated or are in the process of replicating at the time of exposure to these agents (Latt and Loveday, 1978). Further evidence for the association of SCE formation and replication was presented by Kato (1980) who artificially manipulated the S phase length and the number of replication forks with FUdR. Kato's findings corroborate the notion that SCE is rendered more likely by the presence of large numbers of replication forks (Kato had also advanced this idea in two of his earlier papers, 1977b,c). Painter (1980) suggested that in addition to being replication fork restricted, the process of SCE is restricted to the junctions between replicon clusters. Painter argued that

> damage to DNA that involves slowing or stopping of growing points disrupts the timing of the program for replication of contiguous clusters so that the DNA in junctions remains unreplicated for long times. This increases the probability of double strand breaks after one cluster has finished replication. If this happens, both a daughter and a parental strand of each polarity will be available for ligation with the DNA of the unreplicated adjacent cluster, and there would be a finite probability that the daughter strands, rather than the parental ones will join with the unreplicated strands. When the replication of the other clusters finishes, the normal ligation process at the junction will be complete. This exchange requires only one double strand break in the parental strands, which is consistent with data showing that the production of SCEs is a linear function of dose (Carrano et al., 1978, Perry and Evans, 1975). That is SCE results from a single hit even though two double stranded molecules participate in each exchange. Agents that block chain elongation will often cause DNA in clusters to remain partly replicated for long times. Agents like X-rays, which block the initiation of whole clusters, will rarely do this.
>
> Painter (1980, p.339)

The attractive feature of this model is that not all replication forks which encounter a lesion are subject to SCE events: only junctions between replicated and unreplicated clusters of replicons are areas in which SCE is likely to occur (and therefore are a subset of the set of all replication forks). The number of potential SCEs per genome therefore decreases to a more conceptually manageable level.

In 1980 Ishii and Bender (independently from Painter) propounded a "replication detour" model for SCE. Their model is similar to Painter's model in that it does not involve branch migration nor recombinational repair (see Figure 1.6). These authors suggest that a gyrase-like enzyme or unwinding protein might be responsible for the generation of SCEs. Ishii and Bender, unlike Painter, put no restriction on the type of replication fork subject to SCE. Figure 1.6 shows some of the main models for SCE.

In conclusion, SCE is induced by a wide variety of agents which modify DNA in very different ways. Although the mechanism of SCE has not yet been established, it is clear that SCE is, among other things, an S-dependent process in which double strand polarity-restricted exchange occurs between sister chromatids.

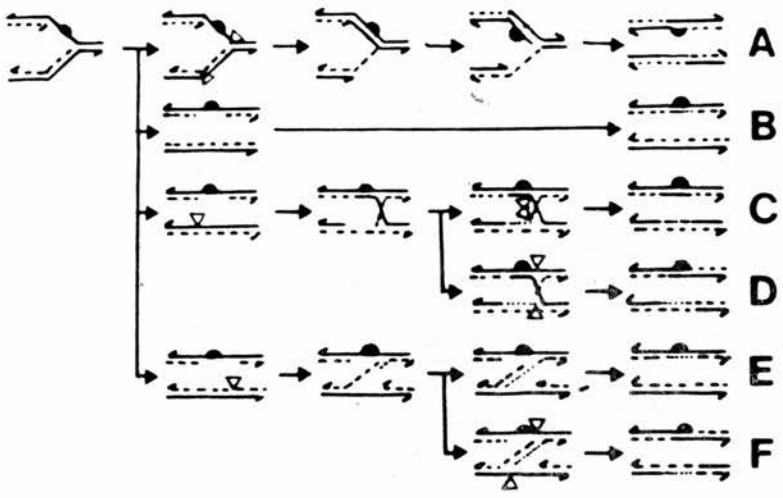


Figure 1.6A. Models for SCE Formation (From Ishii and Bender)

A. Replication detour model of Ishii and Bender (1980)

B. Post-replication repair by gap-filling

C. and D. Recombinational repair

E. and F. Replicative bypass repair through branch migration.

Solid lines indicate non-substituted DNA, broken and dotted lines indicate BUdR-substituted DNA, semi-circles indicate damage and triangles indicate nicks.

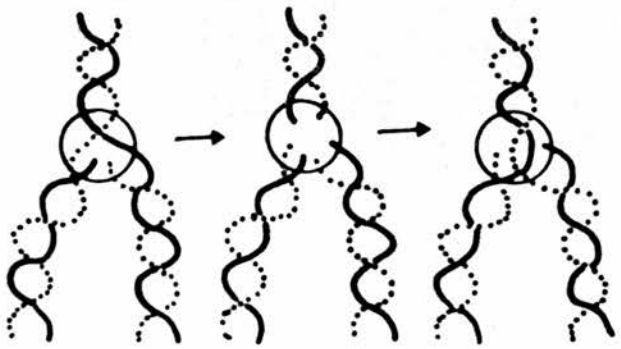


Figure 1.6B. Painter's Model for SCE (1980).

Double-strand recombination at replicon cluster junctions. The exchange is between the newly formed strands of the replicated cluster and the unreplicated cluster's parental strands. (After Painter, 1980)

CHAPTER 2

MATERIALS AND METHODS

A. Cells used in experiments and growth conditions for the detection of SCE

(i) Human peripheral blood lymphocytes

Heparinized venous samples were drawn from donors immediately prior to the onset of the experiment. Occasionally it was necessary to delay setting up the experiment. In such rare instances the samples were stored at 4°C for no longer than 14 hours before stimulation with phytohemagglutinin (PHA).

Whole blood (0.8 ml) was added to 9.2 ml of RPMI 1640 medium supplemented with 15% fetal calf serum, 1% reconstituted PHA (Wellcome HA15) and 2.5 x 10^{-5}M BUdR (final concentration). The cells were grown at 37°C in black boxes to avoid photolysis of the BUdR-substituted DNA (Ikushima and Wolff, 1974).

(ii) Chinese hamster ovary cells (CHO)

CHO cells were maintained by 2-3 passages per week. The fibroblasts were grown in a 5% CO_2 incubator at 37°C. Every 3-9 months the old stocks were replaced by CHO cells recovered from liquid nitrogen.

CHO fibroblasts were cultured in HEPES-buffered McCoy's 5A medium supplemented with 15% fetal calf serum. A final concentration of 1 x 10^{-5}M BUdR was added to the experimental cultures. CHO cells were grown in 25 or 75 cm^2 Falcon flasks. Care was taken to ensure that cultures would be near-confluent by harvest time in order to

maximize the yield of mitotic cells. The CHO cells used (derived from the parental CHO line initiated by Puck in 1957, J. Exp. Cell Med. 108: 945, 1958) have a stable karyotype of 20-21 chromosomes. In the culture conditions employed the cells remained diploid and the cell cycle was approximately 11-12 hours.

B. Harvest procedures

(i) Peripheral blood lymphocytes

Colchicine(5×10^{-7}M final concentration) was added for the last 2½ hours of culture. At 72 hours the cells were spun down and treated at room temperature with 0.075 M KCl for 8 minutes, spun down again and fixed with three washes of glacial acetic acid-methanol (1:3). The slides were air dried and allowed to age for 3 days before staining was carried out.

(ii) CHO sequential harvests

Colcemid (2×10^{-7}M final concentration) was added 22 hours after the addition of BUdR. At 24 hours the mitotic cells were collected by shake-off, spun down and treated at room temperature with 0.075 M KCl for 3 minutes, spun down again and fixed once in glacial acetic acid-methanol (1:3). Fresh medium (pre-warmed) containing BUdR and colcemid was added to the cultures and these were re-incubated until the second harvest (26 hours) at which time the above procedure was repeated. The third harvest was done at 28 hours. Slides were air dried or flamed and stained immediately or on the following day.

C. Staining procedure

A modification of the fluorescence plus Giemsa technique of Perry and Wolff (1974) was used to obtain Giemsa-stained "harlequin" chromosomes. This modified procedure is outlined on the following page.

Time	Slide Treatment	Notes
15 min	Hoechst 33258, 0.5 x 10^{-6} g/ml double distilled water	1
	Rinse in deionized water	
	Mount in double distilled water	2
40-60 min	UV exposure in a UV-box illuminated by Philips TL20W/80 F20T12BLB tubes	3
	Remove coverslips	
20 min	SSC at 60°C (SSC consists of 1.753 g of sodium chloride and 0.882 g of trisodium citrate dissolved in 1 l of double distilled water).	
	Rinse in deionized water	
2-3 min	4% Giemsa made up in pH 6.8 Gurr's buffer	
2-3 hours	Dry	
10 min	Xylene	
	Mount in DePX	

Notes:

1. For CHO slides optimal staining was obtained by using a 15 minute treatment in 5 x 10^{-6} g/ml Hoechst 33258.
2. pH 6.8 Gurr's phosphate buffer may be used instead of double distilled water.
3. UV exposure time is critical. Optimal staining is achieved by exposure to UV light for a period of time which in practice varies from one occasion to the next. It is best to try first a test series with varying UV exposure times and then select the best one to use for staining the rest of the slides.

D. Scoring

All slides were coded and randomized in order to minimize observer bias. Twenty metaphases were scored from each culture. Occasionally, because of gross delay in division, it was not possible to score as many as twenty cells, this is footnoted in the tables of results. When sequential harvests were performed only one harvest was picked for scoring. Only complete (46 chromosome) lymphocyte metaphases were scored. CHO metaphases with 20 or 21 chromosomes were scored.

E. Handling of hazardous chemicals and mutagen contaminated cultures

Any substance which is potentially dangerous must be treated with as much caution as a known mutagen and/or carcinogen. For the sake of simplicity such substances will be referred to as "mutagens".

All manipulations involving mutagens were carried out in a Microflow vertical laminar flow safety cabinet except in cases where it was necessary to use the 37°C hot-room in order to avoid cell cycle delay caused by lengthy washing out procedures (Chapter 3).

Mutagens were weighed out to the nearest 100th of a mg on an Oertling R52 balance. All mutagens that were soluble in water were dissolved in sterile distilled water just before use. Those chemicals which were insoluble in aqueous solutions were dissolved in dimethylsulfoxide (DMSO, Koch-Light). Final concentrations of the mutagens were added to the cultures with a Gilson P200 automatic pipette (0.25% instrument accuracy). DMSO concentration in the culture medium did not exceed 0.5% (except in one experiment, Chapter 4.4).

When it was necessary to remove the mutagen from the cultures, the cells were washed 3 times with 10 ml phosphate buffered saline containing BUdR. This procedure was carried out in subdued light (Ikushima and Wolff, 1974).

F. Mutagens used in experiments

Name	Abbreviation	M.W.	Solvent	Manufacturer/Supplier
Anthramycin	ANT	315.3	DMSO	Hofmann la Roche (gift)
Bleomycin	BLM	1400	H_2O	Lunbeck
Ethyl methanesulfonate	EMS	124.2	H_2O	Sigma
Ethyl nitrosourea	ENU	117.1	DMSO	Fluka
Hydralazine	HYD	196.6	H_2O	CIBA
Methyl methanesulfonate	MMS	110.1	H_2O	Aldrich
Mitomycin C	MMC	334.3	H_2O	Kyowa

G. Statistical analysis

Statistical analysis of the data was performed using analyses suggested by Drs. Andrew Carothers and Anthea Springbett, statisticians at the MRC Clinical and Population Cytogenetics Unit, Edinburgh; these are detailed with the relevant experiments.

H. Special procedure (for Chapter 4.1): CHO synchronization following an adaptation of the method described by Petersen et al. (1968).

(i) Preparations for the procedure

During the week prior to the procedure cell-free "conditioned medium" (CM) was collected from stock cultures of CHO cells. After culturing CHO cells for approximately 24 hours the medium in which they were grown can be recovered and used again. This medium, CM, is recommended for mitotic shake off procedures. CM was stored at 4°C for no longer than 1 week and was warmed to 37°C prior to use.

(ii) Pre-mitotic cell collection procedure

The following steps were performed in order to eliminate any dead cells and cellular débris from the monolayer.

1. The near-confluent culture flasks were gently agitated
2. The medium was discarded and replaced by warm CM
3. The flasks were incubated for 15 minutes
4. Steps 1-3 were repeated and immediately followed by the first step of the mitotic shake off for collection procedure.

(iii) Mitotic shake off for collection procedure

1. The culture flasks were rocked gently but quickly
2. The medium from the flasks was decanted into centrifuge tubes (cold)
3. and replaced by warm CM

4. The cultures were incubated for 15 min at 37°C
5. The mitotic cells from the shake off were spun down at 4°C
6. The supernatant was poured off and discarded
7. The mitotic cells were resuspended in a small volume of cold medium
8. The shake off yield was counted with a hemocytometer
9. A slide was prepared from the shake off products to determine the mitotic index
10. The foregoing steps were repeated every 15 minutes until the desired number of cells had been collected.

Approximately 6 x 10^5 cells were collected per 75 cm^2 flask per shake off. Mitotic indices from all shake offs exceeded 90%. The entire procedure was performed in subdued light conditions because 2/3 of the cultures had already undergone one round of replication in BUdR (See Chapter4.1, Experimental Design).

CHAPTER 3

THE EXCHANGE HYPOTHESIS FOR THE FORMATION OF CHROMATID ABERRATIONS: THEORETICAL CONSIDERATIONS AND AN EXPERIMENTAL TEST

Introduction

Most conventional accounts of the induction of chromatid aberrations (particularly by X-rays) include the two chief hypotheses proposed for the formation of chromatid aberrations: the breakage-first hypothesis and the exchange hypothesis. Seldom is an evaluation of these hypotheses given on the basis of experimental evidence accumulated since they were advanced. The latter statement is not meant as a criticism of those who have written about these hypotheses. Indeed it is a brave person who attempts to extricate a general story from the myriad of seemingly disparate results obtained on the subject. The proponent of the exchange hypothesis, Revell, wrote rather dejectedly in 1974:

> I am aware that many have despaired of understanding the problem and are leaving the few protagonists to settle its esotericisms among themselves, in the hope that these few will hand out an agreed doctrine which is comprehensible to all. This is not a healthy state for any branch of research to be in.

The present account of the theories on the formation of chromatid aberrations in no way professes to be a complete one. The mechanism(s) proposed so far for the formation of chromatid aberrations will be discussed in as much as they are pertinent to the theme of this thesis, sister chromatid exchange.

At this point a review of the breakage-first and the exchange hypothesis is in order. Since these hypotheses were originally proposed to account for the existence of chromatid aberrations in cells subjected to radiation, the discussion will initially centre around radiation-induced damage.

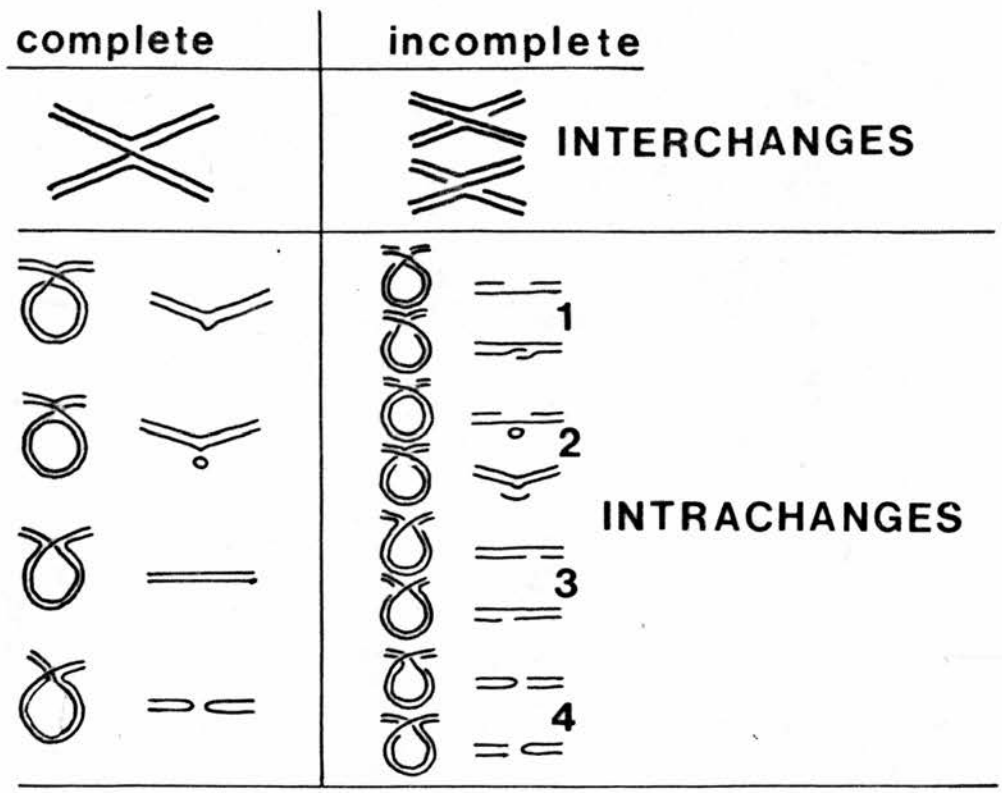
The breakage-first hypothesis, though originally suggested by Stadler in 1932, was developed and formalized by Sax in 1938. The breakage-first hypothesis regards observed metaphase breaks as the result of single-hit events which sever DNA stands. The broken DNA ends may rejoin normally, may become involved in an exchange or remain unrepaired. Radiation is viewed essentially as a breaking agent and the cell is left with the task of dealing with the damage. Exchanges therefore arise as a consequence of reunion of DNA strands after breakage.

In the exchange hypothesis however, radiation is not viewed as the primary breaking agent. In 1954 Revell advanced the theory that radiation initiates an exchange event in the necks of loops of chromosomal DNA. This exchange, if incomplete, gives rise to chromatid breaks. The exchange hypothesis therefore regards a faulty process of exchange as the primary cause of breakage.

> If it could be shown that all chromatid changes observed at metaphase arise as the exchange theory proposes, we would be left with no mitotic evidence that radiation has caused chromatid breaks anywhere except where it has also caused chromatid rearrangement.
>
> (Revell, 1974, p. 389)

Figure 3.1 illustrates the formation of chromatid aberrations according to Revell's hypothesis.

At first sight it might seem possible to design experiments which would establish which hypothesis, if either, is more accurate in its predictions. Unfortunately such endeavours have been blocked by disputes over the question: what is a break, or more to the point, what is not a break (Revell, 1959, 1963)? Ionizing radiation produces a large number of constrictions and gaps which do not leave fragments


complete
incomplete
INTERCHANGES
1
2
3
4
INTRACHANGES

at anaphase as Revell (1959) clearly demonstrated. According to Revell (1959) the frequency of chromatid breaks produced by ionizing radiation had been grossly overestimated. In his experiments on Vicia faba only 32 out of 587 "conventional breaks" showed up as acentric fragments at anaphase. In this connection it is interesting to note that Revell (1954, 1959) presented evidence that some, albeit not all, "gaps" (these chromatid aberrations were not regarded by Revell as true breaks) were involved at exchange sites. This of course raised questions about chromatid gaps.

Scott and Evans (1967) studied cell-cycle dependent changes in the response of V. faba cells to X-ray induction of aberrations. Scott and Evans failed to observe the 5:2 ratio of chromatid breaks to isochromatid breaks and found this ratio to vary depending on the phase of the cell cycle at the time of irradiation (for derivation of 5:2 ratio see figure 3.1). These authors concluded that all aberrations (except gaps) were "a consequence of exchange following a process of mis-repair of primary lesions that are not breaks". Chromatid gaps, according to Scott and Evans, could be taken as the cytological manifestation of non-repaired X-ray induced primary DNA lesions.

In 1969 Evans and Scott studied the induction of chromatid aberrations by nitrogen mustard (in 1958 Revell had extended his hypothesis to encompass chromatid aberrations induced by chemicals). These authors found that, although nitrogen mustard damaged DNA irrespective of cell phase at the time of treatment, structural changes (aberrations) were produced only after cells had undergone DNA synthesis. Evans and Scott failed to observe the expected 5:2 ratio of chromatid breaks to isochromatid breaks; rather, they found significantly fewer chromatid breaks than incomplete isochromatid aberrations. Evans and Scott concluded that, in cells exposed to nitrogen mustard during the S phase, isochromatid breaks were the result of mis-replication at a single locus and not the result of an exchange between two loci (one on each chromatid) as Revell

had envisaged.

The opportunity to reveal exchanges at the sites of chromatid breaks arose with the development of techniques to visualize SCE. The term SCE now connotes a process which is intimately associated with DNA replication (see Chapter 1). For the sake of brevity "SCE" will be used in this Chapter to convey the broader concept of BUdR-label exchange between sister chromatids and will thus encompass replication-fork-independent exchange processes between sister chromatids.

CHO cells irradiated in the last G2 (pre-fixation) phase do not exhibit an increase in SCE frequency (Perry and Evans, 1975) whereas chromatid breaks are abundant in such cells (e.g. Natarajan et al., 1980a). It may be inferred from the discrepancy between the number of complete exchanges (SCEs) and incomplete exchanges (chromatid breaks) induced by X-rays in G2 cells that Revell's exchange hypothesis cannot account for the formation of breaks in these cells.

Heddle et al. (1969) performed an autoradiographic study in which they labelled one DNA strand with tritiated thymidine. They used X-rays to induce breaks and looked for label switches at the break sites. They had estimated that 40% of the breaks should be associated with label exchange if all breaks were the result of incomplete exchange (as Revell had hypothesized, see Figure 3.2.), and obtained results which closely matched this expectation. However, because their samples were small the experiment was repeated and this time Heddle and Bodycote (1970) found far fewer breaks to be associated with label exchange. Although they recognized that some breaks might have arisen as a consequence of incomplete exchange, Heddle and Bodycote concluded that Revell's model alone could not account for the formation of all breaks.

Following the development of the FPG technique, a number of experiments was performed on radiation damaged cells to determine the incidence of breaks associated with exchange (Ikushima, 1977, Wolff and Bodycote, 1975). Again it was found that the exchange hypothesis could not account for the formation of all lesions observed although it could account for some. Further evidence in support of this conclusion may be extracted from experiments with UV, ionizing radiation and chemicals which were not specifically designed to test Revell's hypothesis (Kihlman et al., 1977, Natarajan and Obe, 1978, Natarajan et al., 1980b, Shiraishi et al., 1979).

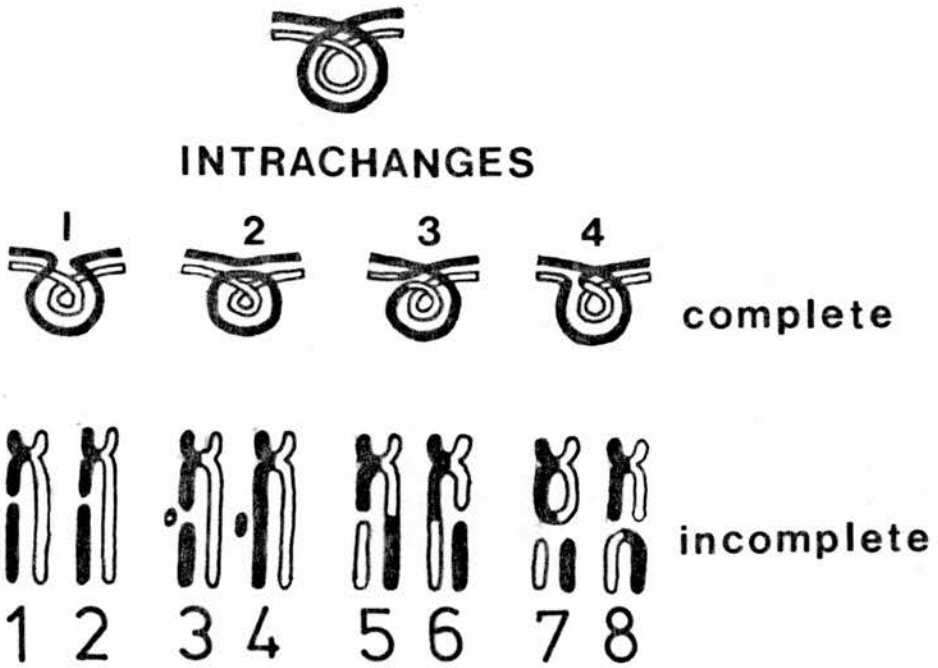


Figure 3.2. Formation of Chromatid Aberrations According to the Exchange Hypothesis and Detection of Sister Chromatid Exchange at the Break Site.
Exchanges 7 and 8 cannot be scored for SCE with certainty, type-4 aberrations will be missed in the course of scoring (see Fig. 3.1.), this leaves five types of aberrations which may be scored for SCE (type-1,2,3,5,6), two of which will be coincident with exchange (40%). The solid regions indicate unifilarly BUdR-substituted strands. Figure modified from a diagram published in the paper by Heddle et al. (1969).

On the other hand, support for the Revell hypothesis comes from experiments on the induction of SCE and aberrations in "hot spots" of rat chromosomes. Ueda et al.(1976) claimed as support for Revell's hypothesis the observation that SCEs induced *in vitro* by dimethylbenz[a]anthracene (DMBA) occurred at the same late-replicating regions as DMBA-induced aberrations in rat bone marrow cells *in vivo*. Their conclusion is less than convincing on several counts: they did not investigate the coincidence of break-points and SCEs in the same cells; the control (i.e. untreated) level and distribution of SCE *in vitro* is not quoted and so the number of SCEs induced by DMBA is unknown; under the experimental conditions employed (treatment of cells for the final 6 hours before fixation) DMBA is known to cause aberrations preferentially in late-replicating heterochromatic regions of the rat genome (Sugiyama, 1971). Since SCE cannot be induced in DNA that has already completed synthesis (Latt and Loveday, 1978) any DMBA-induced SCEs must also occur in the late-replicating regions. It is therefore not surprising that both aberrations and SCEs appear to occur preferentially in late-replicating regions and this evidence, as it stands, cannot be counted as evidence favouring the exchange hypothesis (but, see Discussion).

It was of interest to investigate the frequency of aberrations coincident with SCE in cells treated at various stages of their second cell cycle in an attempt to uncover some aberrations à la Revell. Bleomycin (BLM) was used as the clastogenic agent because, like X-rays, it causes predominantly chromatid-type aberrations in the first post-treatment mitosis of cells exposed in G2 (Natarajan and Obe, 1978, Dresp et al., 1978, Paika and Krishan, 1973). Bleomycin is a complex mixture of compounds produced by *Streptomyces verticillus* (Umezawa, 1975). Several very comprehensive reviews have been written on its biochemical and cytogenetic effects (Umezawa, 1975, Vig and Lewis, 1978). Briefly, BLM's primary target is DNA and BLM does not affect the synthesis and function of mRNA (Kuo et al., 1977).

DNA synthesis initiation and termination in cells exposed to BLM occur at normal rates (Tobey, 1972). BLM causes single strand scission of DNA (Fujiwara and Kondo, 1973, Iqbal et al., 1976, Miyakani et al., 1971, Natarajan and Obe, 1978, Suzuki et al., 1969). The clastogenic effect of BLM is manifested at the cytogenetic level by the presence of predominantly chromatid-type aberrations induced in G2 cells Dresp et al., 1978, Natarajan and Obe, 1978, Paika and Krishan, 1973). Although some chromatid-type aberrations are observed after G1 BLM exposure, suggesting an S-dependent component of BLM damage (Tamura et al., 1974), GÒ and G1 treated cells are characterized mainly by chromosome aberrations whose frequencies are linear with BLM dose (Dresp et al., 1978, Tamura et al., 1974). In these respects BLM acts much like X-rays, that is BLM causes cytogenetic damage in an S-independent non delayed fashion. Like X-rays, BLM does not induce many SCEs (Gebhart and Kappauf, 1978, Lambert et al., 1978, Perry and Evans, 1975) and this fact circumvents the problem of having to estimate random coincidence of SCEs and breaks which ought to be done if many SCEs are induced in each cell.

The experiments were carried out on human peripheral blood lymphocytes which, because of their stable karyotype, are satisfactory for scoring. Synchronized CHO cells would have been better target cells to use in order to ensure that the majority of cells were in a similar stage of the cell cycle. However, CHO cells exhibit too high a background aberration frequency for the purposes of this study.

Materials and Methods

Human peripheral lymphocytes were cultured as described in Chapter 2. The setting up and harvesting of the cultures were staggered (2 hour intervals) so that all the cells could be exposed to BLM at the same time. BLM final concentrations of 5×10^{-5}M and 1×10^{-5}M were administered for a two-hour period 18, 16, 14, and 12 hours before harvest. At the end of the treatment, the suspensions were spun down

and the cells were washed three times in 37°C complete medium, resuspended in fresh complete medium (BLM-free) and incubated until harvest. All of these operations were carried out in a 37°C hot-room in order not to delay cell growth. A yellow safe-light (Kodak OB filter) was used in order to avoid photolysis of the BUdR-substituted DNA strands (Ikushima and Wolff, 1974).

Criteria for scoring aberrations

Single chromatid breaks, single chromatid gaps and isochromatid gaps were scored. Achromatic lesions smaller than the width of a chromatid and continuous with the chromosome axis were classified as chromatid or isochromatid gaps. Displaced fragments and fragments aligned with the chromosome axis but separated from it by a distance greater than the width of a chromatid were classified as chromatid breaks. Isochromatid breaks were not scored because of the uncertainty involved in scoring for SCE at the break point.

Results

The results are summarised in tables 3.1 and 3.2.

Table 3.1. Incidence of BLM Induced Gaps and Breaks With and Without SCE

t	[BLM]	n	SCE ± S.E.	CG	CB	IG	T	COINCIDENCE
10-12	$5x10^{-5}$	53	7.9 ± 0.40	19	29	2	50	16%
	$1x10^{-5}$	131	8.9 ± 1.30	19	27	4	50	24%
12-14	$5x10^{-5}$	46	8.5 ± 0.59	29	21	0	50	12%
	$1x10^{-5}$	74	7.7 ± 0.44	25	22	3	50	20%
14-16	$5x10^{-5}$	47	8.6 ± 0.55	21	30	1	52	17.3%
	$1x10^{-5}$	84	7.3 ± 0.33	15	34	2	51	19.6%
16-18	$1x10^{-5}$	140	7.7 ± 0.25	16	27	3	46	17.4%
Control		100	7.3 ± 0.27	1	4	0	5	2/5

t denotes time of exposure (hours before harvest); n, the number of cells analysed; S.E., the standard error; CG, chromatid gap; CB, chromatid break; IG, isochromatid gap, T, total number of aberrations scored.

A $5x10^{-5}$M concentration of BLM administered 16 to 18 hours before harvest caused too much delay for aberrations to be scored in sufficient numbers.

Analysis of the data presented in Table 3.1.

The observed percent coincidence of aberrations and SCEs deviates significantly from the expected 40% ($p<0.001$, $X^2= 86.45$)

Cells in which aberrations were accompanied by SCE (n=59) had a mean SCE frequency of 8.86 (S.E.=0.45). Cells in which aberrations were independent of SCE (n=156) had a mean SCE frequency of 7.74 (S.E.=0.27). The difference of means is significant at the 5% level (t=2.18).

Table 3.2. Incidence of BLM Induced Aberrations With SCEs

t	[BLM]	CG*	ε	CB*	ε	IG*	ε
10-12	$5x10^{-5}$	3/19	15.8%	4/29	13.8%	0/2	0%
	$1x10^{-5}$	0/19	0%	9/27	33.3%	2/4	50%
12-14	$5x10^{-5}$	1/29	3.4%	5/21	23.8%	0/0	-
	$1x10^{-5}$	2/25	8.0%	8/22	36.4%	0/3	0%
14-16	$5x10^{-5}$	2/21	9.5%	7/30	23.3%	0/1	0%
	$1x10^{-5}$	3/15	20.0%	8/34	23.5%	0/2	0%
16-18	$1x10^{-5}$	0/16	0%	8/27	29.6%	0/3	0%
Control		0/1	0%	2/4	50.0%	0/0	-

t denotes time of exposure to BLM (hours before harvest);
CG*, number of chromatid gaps with SCE/total number of gaps;
CB*, number of chromatid breaks with SCE/total chromatid breaks;
IG*, isochromatid gaps with SCE/total isochromatid gaps;
ε, percent coincidence of association of each type of aberration with SCE.

Analysis of the data presented in Table 3.2.

From the results presented in Table 3.2., it is clear that there is no consistent association of SCE with any of the aberration types scored. However, chromatid breaks/SCEs are significantly more associated than chromatid gaps/SCEs ($p<0.005$, $X^2=19.4$). The difference between the association of chromatid breaks/SCEs and isochromatid gaps/SCEs is not significantly different at the 5% level ($X^2=1.2$). Although chromatid breaks are more frequently associated with SCE than other types of aberrations, the percent association of chromatid breaks and SCEs is still lower than the predicted 40% (5% confidence level for X^2 on pooled data for chromatid breaks).

The results of this study indicate that only a small percentage of BLM-induced breaks are associated with SCE (Table 3.1.). The frequency of association (12-20%) of these events is significantly lower than the expected (40%) if all breaks were the result of incomplete exchange. If one subdivides the aberrations scored into the three categories listed in Table 3.2., the percent association of chromatid breaks, chromatid gaps and isochromatid gaps with SCE is still significantly lower than the expected 40%. It is interesting to note, however, that significantly more chromatid breaks are coincident with SCE than are chromatid gaps. This observation fits in with the notion that gaps and breaks cannot be classified under the same category. (Revell, 1959, 1963, Scott and Evans, 1967). Further, because it is difficult to distinguish some gaps from breaks, the real frequency of association between breakage and exchange may be higher because of misclassification of gaps as breaks.

The question that really needs answering is what proportion of chromatid breaks are the result of incomplete exchange? There are two ways of obtaining association of breakage and exchange at the same site: (1) random association of break points with SCE sites (2) failure to complete the exchange. As was mentioned in the Results section, the mean SCE frequency of cells in which at least one break was coincident with exchange was found to be significantly higher than the mean SCE frequency of cells in which breaks appeared but were independent of SCE sites. However, since the difference of means is of the order of 1 SCE per cell, one must be cautious in attributing association of breakage and exchange to chance alone.

Kato (1977b) described an experiment in which he exposed uni- and trifilarly BUdR substituted CHO chromosomes to fluorescent light. When he exposed cells in S phase to fluorescent light, he found chromatid deletions associated with SCE with frequencies of approximately 45% and 25% in uni- and trifilarly substituted chromosomes respectively. Since fluorescent light causes photolysis of BUdR-substituted DNA (Ikushima and Wolff, 1974), Kato argued that the apparently low value of 25% for associated deletions and ex-

changes could have been caused by a swamping effect of breaks induced in post-replication DNA in trifilarly substituted chromosomes. That is, the trifilarly substituted chromosomes were subject to replication fork (and perhaps SCE) independent breaks, the high incidence of such breaks leading to a reduction in the relative frequency of SCE-associated breaks. Other findings in Kato's study tend to substantiate his proposal: he noted that cells irradiated in G2 essentially lacked SCE-break associations. In the light of his findings one might expect that the association between SCE and chromatid aberrations should decrease as time of exposure to BLM approaches fixation time (i.e. as BLM hits cells that are closer and closer to second mitosis). This expectation was not met by the findings of the present study. Failure to observe a change in SCE-break associations with varying time of exposure could be attributed to the heterogeneity of the cell population studied.

It is interesting to note that cultures exposed to $5x10^{-5}$M BLM consistently showed lower coincidence of SCE and breaks than cultures exposed to a BLM concentration of $1x10^{-5}$M. This trend could not be analysed statistically but is worth mentioning because it fits rather well with Kato's idea of swamping of incomplete-exchange-induced aberrations by aberrations caused by strand breaks in post-replication DNA. This is, of course, mere speculation, and it is unfortunate that time did not permit further investigation of this question.

After the completion of this study Galloway and Wolff (1979) published results which led them to conclude that Revell's exchange hypothesis could not account for the formation of all chromatid breaks. Another paper appeared in 1980 in which van Kesteren-van Leuwen and Natarajan re-examined the results of Ueda et al. (1976, see Introduction). These authors used an improved experimental approach and showed that 46.9% of DMBA-induced breaks are coincident with SCE. van Kesteren-van Leuwen and Natarajan followed Ueda et al.'s protocol and treated rats with DMBA 6 hours before killing and obtained cells which they analysed

for the simultaneous occurrence of SCEs and aberrations. These authors were quite cautious in interpreting their results and suggested that the effect they picked up might be explained by the fact that they were considering exclusively heterochromatic regions. They suggested that these regions, rich in repetitive DNA and late-replicating, might be of particular interest when trying to verify the predictions of Revell's exchange hypothesis since Revell's observations were taken from a cell system (V. faba) which is rich in large blocks of late-replicating DNA. It is worth pointing out again that Latt and Loveday (1978) observed that the frequency and location of SCEs induced at different times during S is restricted to those regions which have replicated during or after DNA damage.

In conclusion, it is evident from a literature survey and the results presented in this chapter, that Revell's exchange hypothesis cannot account for the formation of all chromatid breaks. However, the observed association of chromatid breaks with SCE points to some involvement of incomplete exchange in the induction of chromatid breaks. The degree of association of these two events seems to be related to the state of the cell at the time of injury.

CHAPTER 4

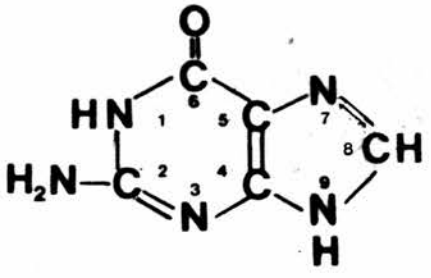
MOLECULAR NATURE OF LESIONS INVOLVED IN SCE INDUCTION

General Introduction to Chapter 4

A bewildering number of agents induce SCEs. Biochemical studies indicate that the lesions produced by each SCE-inducing agent vary both qualitatively and quantitatively. Many studies have been devoted to the analysis of known DNA repair mechanisms and SCE induction with a view to establishing a model for the generation of SCE. Arguably SCE could result from a fairly non-discriminating lesion-avoiding mechanism: that is, in terms of this model, the nature of the lesion would be less important than the fact that it is there at all. Indeed this would be the simplest explanation for the SCE-induction end-point. However, this view of SCE-induction is unsatisfying with respect to its predictive abilities. Ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) are both SCE-inducing agents. However, MMS is a more potent SCE-inducer on a molar basis than EMS (Perry and Evans, 1975). Differences in the rate of cellular up-take of these two componds are unlikely to account for this discrepancy since both chemicals are of low and similar molecular weight (124 for EMS and 117 for MMS). One could argue that the biochemical half-life of these compounds could account for differences in their ability to induce SCEs since EMS has a half-life of 2.2 hours and MMS has one of 8.5 hours (Jensen et al., 1977). However, biological half-life cannot be the only factor because N-methyl-N-nitrosoguanidine (MNNG), which has a biological half-life of 14 minutes (Jensen et al., 1977), induces approximately the same number of SCEs as EMS but at concentrations 1000 timeslower than EMS concentrations (Perry and Evans, 1975).

One is therefore left with the rather unsettling thought that SCE could be the product of certain types of lesions. Mutagens cause different kinds and different amounts of DNA lesions because of differences in their affinity for particular DNA binding sites. A considerable amount of information on the interaction of DNA and DNA-damaging agents may be obtained from Roberts (1978), Pegg (1977), Singer (1975) and Lawley (1972a,b).

Many studies have been devoted to trying to establish a link between specific alkylation products and mutagenesis. Initially it was thought that the alkylation of guanine at the N-7 position, which can sometimes account for up to 90% of alkylation reactions (Roberts, 1978), could be important in mutagenesis. However, following Loveless' suggestion (1969) that alkylation at the O-6 position of guanine could result in mis-pairing (GC⟶AT transitions) an enormous number of experiments were performed which led to the now generally accepted notion that O^6-alkylguanine (O-6-alkylG) is a more important lesion than N-7-alkylG in mutagenesis, and perhaps carcinogenesis.



GUANINE

Lawley and Orr (1970) showed that in E. coli O-6 alkylation products of MNNG were specifically excised whereas N-7 alkylation products were not. They suggested that the repair machinery might recognize and excise potentially mis-pairing modified bases. Couldre and Miller (1977) showed that MNNG and EMS produce GC⟶AT transitions in E. coli. In 1978 Metah and Ludlam, using artificially synthesized copolymers, poly(dC, m^6dG) obtained mis-incorporation of UMP in the product RNA polymer.

With regard to mammalian systems, Goth and Rajeway (1974) obtained results from which they suggested that the specificity of ethyl nitrosourea (ENU) in inducing brain tumors could be correlated with the very slow rate of removal of O-6-ethylG products in brain tissue as compared with other tissues in which tumors are rarely observed. O-6-methylG products of methyl nitrosourea (MNU) also persist for a long time in the brain (Kleihues, 1977). Magee et al. (1975) also suggested that the fact that single large doses of ENU and DEN can induce tumors in kidney and brain but not in liver can be accounted for by differences in the rates of excision of O-6-alkylG products from these tissues (excision being fastest in the liver, intermediate in the kidney and slowest in the brain). Frei et al. (1978) further reported that O-6-alkylG was removed more rapidly from the liver than from brain, kidney and lung tissues. At this point it should be mentioned that the excision of O-6-alkylG in mammalian cells is probably achieved by a demethylase rather than by an N-glycosidase as has been suggested for E. coli (Pegg, 1978). One might well wonder what all of this has to do with SCE induction. The link between O-6-alkylation of guanine and SCE was established when Goth-Goldstein (1977) showed that ENU and MNU alkylation products at the O-6 position of guanine are not excised in xeroderma pigmentosum cells whereas N-7-alkylG is lost from these cells at the same rate as in normal cells. This finding coupled with the fact that xeroderma cells are more sensitive to SCE induction by a spectrum of chemical mutagens than similarly treated control cells (Wolff et al., 1977) has led to the implication of O-6-alkylG in the induction of SCE (Wolff, 1978a,b). The induction of SCE by mitomycin C, which does not alkylate the N-7 but does alkylate the O-6 positions of guanine (Tomasz, 1974), further supports the view that some SCEs are caused by O-6 alkylation of guanine (Carrano et al., 1979).

The experiments included in this chapter deal with SCE induction by single mutagens and pairs of mutagens. These experiments were designed to determine whether the extent of alkylation of the O-6 position of guanine can be

correlated with induced SCE frequencies (comparisons between single mutagens). Moreover, SCE induction by pairs of mutagens was studied to investigate whether the mutagens in each pair would interact in inducing SCE (blocking, perhaps through competition for O-6 sites, or synergism) or whether their effects would be additive with respect to SCE induction.

CHAPTER 4

SECTION 1: SCE INDUCTION BY COMBINED TREATMENT WITH METHYL METHANESULFONATE AND X-RAYS

Introduction

The bulk of ionizing radiation-induced damage is rapidly repaired (Lett et al., 1967, Regan and Setlow, 1974, Pempree and Merz, 1969, Wolff, 1972) by short-patch repair involving no more than 3-4 nucleotides (Regan and Setlow, 1974). However, the possibility that some ionizing radiation-induced lesions might be excised was raised by results obtained by several investigators (Lennatz et al., 1975, Mattern et al., 1973, Paterson et al., 1976, Remsen et al., 1976). This notion was strengthened by the finding that X-rays induce DNA-protein crosslinks (Fornace and Little, 1977). Whether such lesions are involved in SCE induction by X-rays is a matter of debate. At present there is growing evidence that X-ray induced SCEs are few, if there are any (see Chapter 6). It may be that the putative enzymatically excisable lesions caused by X-rays are too few to make a difference to the base-line SCE frequency and/or are not lesions which induce SCE.

Methyl methanesulfonate (MMS) has been described as an X-ray-like alkylating agent by virtue of the fact that it induces short-patch repair (Regan and Setlow, 1974) and blocks replicon initiation (Painter, 1977, Buhl and Regan, 1973). However, unlike X-rays, MMS also decelerates chain elongation (Buhl and Regan, 1973, Dahle et al., 1978, Painter, 1977, Scudiero and Strauss, 1974). On the assumption that MMS and X-rays might induce similar repair enzymes which could affect the number of SCE-initiating lesions, an experiment was designed to study the effects of combined X-ray and MMS injury on synchronized CHO cells.

Materials and Methods

Chinese hamster ovary (CHO) cells were synchronized according to the method detailed in Chapter 2, section H. The experimental design is illustrated in Figure 4.1.1.

Results

The results are presented in Figure 4.1.1, where the mean SCE frequencies (n=20 cells/culture) are summarised. A statistical analysis of the data is presented in Table 4.1.1. It was not possible to pool data from the first cycle treated cells and the second cycle treated cells because first cycle MMS-induced SCEs were significantly higher than second cycle MMS-induced SCEs. Furthermore, first cycle irradiation-induced SCEs were significantly higher than untreated controls and second cycle irradiation-induced SCEs did not differ from untreated controls.

A t test applied to the data indicated that combined treatment of MMS and X-rays (irrespective of the order in which they were administered) yielded a significantly lower SCE value than expected if treatments of MMS and X-irradiation had been strictly additive. This was true for both the first and second cycle insulted CHO cells.

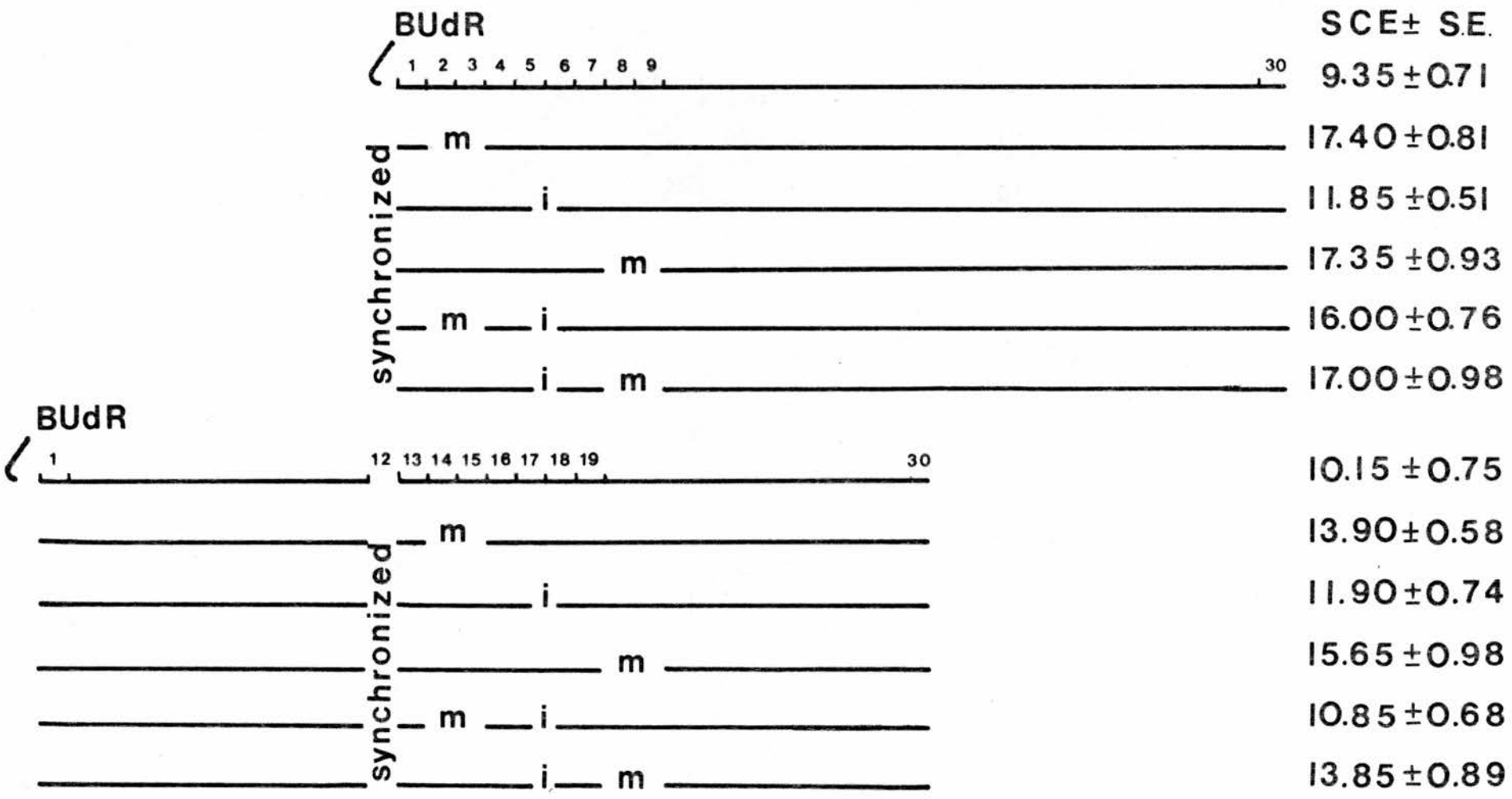

BUdR
1 2 3 4 5 6 7 8 9
30
SCE± S.E.
9.35±0.71
synchronized
m
17.40±0.81
i
11.85±0.51
m
17.35±0.93
m i
16.00±0.76
i m
17.00±0.98
BUdR
1
12 13 14 15 16 17 18 19
30
10.15±0.75
synchronized
m
13.90±0.58
i
11.90±0.74
m
15.65±0.98
m i
10.85±0.68
i m
13.85±0.89

Table 4.1.1. Statistical Analysis of the Data

Cycle	Treatment	SCE	s^2	t	p
1	Control	9.35	10.13		
2	Control	10.15	11.19	0.77	
1	O/i/O	11.85	5.29		
2	O/i/O	11.90	10.94	0.01	
1	Control	9.35	10.13		
1	O/i/O	11.85	5.29	2.85	p<0.05
2	Control	10.15	11.19		
2	O/i/O	11.90	10.94	1.58	
1	MMS/O/O	17.40	13.20		
1	O/O/MMS	17.35	17.40	1.00	
2	MMS/O/O	13.90	6.73		
2	O/O/MMS	15.65	19.19	0.04	
1	MMS(pooled)	17.38	14.91		
2	MMS(pooled)	14.78	13.41	3.09	p<0.01
1	MMS/i/O	16.00	10.84		
1	Expected	19.90	28.62	3.06	p<0.01
1	O/i/MMS	17.00	19.37		
1	Expected	19.85	32.82	2.03	p<0.05
2	MMS/i/O	10.85	11.61		
2	Expected	15.65	28.86	3.74	p<0.01
2	O/i/MMS	13.85	15.71		
2	Expected	17.40	41.32	2.32	p<0.05

Expected SCE values were calculated in the following way e.g. Expected for first cycle MMS/i/O=[MMS/O/O] + [O/i/O] - [O/O/O] = 17.40 + 11.85 - 9.35 = 19.90.

s^2 for the expected is the sum of the s^2 of the three means involved in the foregoing calculation.

-/-/- denotes the sequence of treatments

Discussion

The finding that combined treatment of MMS and X-irradiation induced significantly fewer SCEs than expected is rather surprising. Several explanations may be advanced to elucidate this finding. A recent report (Sono and Sakaguchi, 1981) indicates that the protein synthesis inhibitors cyclohexamide and puromycin decrease the frequency of EMS and MMS induced SCEs. Kato (1980) also found that cyclohexamide completely inhibited SCE induction by fluorescent light. MMS reacts extensively with cellular constituents other than DNA (Roberts et al., 1971) and slows protein synthesis (Fox and Fox, 1967). The notion that the protein synthesis inhibiting capacity of MMS could account for the observed discrepancy in SCE frequencies is unattractive because a lower than expected SCE frequency was also observed in cells irradiated prior to MMS treatment. One could attempt to explain the present findings by using Painter's model (1980) for the formation of SCE (See Chapter 1, Section D). Painter argued that pre-treatment with an agent which prevents the initiation of replicon clusters (and preferably does not induce SCE, e.g. X-rays) would lower the expected SCE frequency induced by post-treatment with an agent which slows or blocks chain elongation. According to Painter, the role of the inhibitor of replicon cluster initiation would be to delay the onset of replication within that cluster thereby allowing more time for the repair system to operate. In this connection it is interesting to note that MMS first inhibits replicon initiation and subsequently slows chain growth (Painter, 1977, Buhl and Regan, 1973). According to Painter's model MMS could be said to "decrease" its own SCE-inducing capacity. The application of Painter's model to explain the results obtained in this experiment is, however, unsatisfactory because the dose of radiation inflicted on the cells cannot be regarded as high enough to block replicon initiation (Tolomach and Jones, 1977). Therefore the reduction in the observed SCE frequency with X-ray pre-treatment cannot be accounted for.

Another possible explanation for the results obtained is that MMS and X-rays induce repair systems which "cross react"; in other words that some of the MMS repair machinery can act on some X-ray induced lesions and vice versa. Bodell (1977) used micrococcal nuclease to digest repair-labelled nuclei of mouse cells exposed to MMS and found a non-uniform repair of chromatin. He attributed this finding to either non-random DNA alkylation or to the inaccessibility of some damaged sites to repair enzymes. On the assumption that some MMS induced lesions might be inaccessible to repair enzymes it is possible to envisage that some of the X-ray induced repair machinery might be better suited to reach these inaccessible sites. Lennatz et al. (1975) pointed out that not all X-ray induced lesions offer a 3'OH group to DNA polymerase I nor a 3'OH and a 5'phosphate group to ligase and that some exonucleolytic action is required to "clean" breaks. It is possible that MMS repair enzymes might speed up this process. This contention is further supported by the findings of Mattern et al. (1973) who found that CHO cells could excise enzymatically gamma-radiation induced 5,6-dihydroxydihydrothymine. Roberts (1978) has suggested that the enzyme involved in this process could also be involved in the removal of small alkyl groups and this is consistent with the idea that MMS and X-ray repair systems might be linked.

Another explanation which may be advanced to account for the results of this experiment is one based on the adaptive-DNA-repair system. This system has been characterized for bacteria (Samson and Cairns, 1977, Jeggo et al., 1977, Schendel and Robins , 1978, Robins and Cairns, 1979, Karran et al., 1979) and it has been suggested that it exists in mammalian cells (Montesano et al., 1979a,b, Samson and Schwartz, 1980). Briefly, adaptive-DNA-repair is characterized in bateria by enhanced survival of cells "primed" with small doses of MNNG and challenged with a large dose of MNNG or other alkylating agents (see above references). In mammalian cells Samson and Schwartz (1980) showed that pre-treatment of CHO cells with non-toxic MNNG doses resulted in enhanced

survival and reduced SCEs following large challenge doses of MNNG, MNU and ENU. These findings raise the possibility that the lower than expected SCE frequency in MMS and X-ray treated cells might be due to an adaptive-DNA-repair system. However, this interpretation must be regarded with some suspicion because the adaptive-DNA-repair system has been demonstrated using alkylating agents only (X-rays do not fall in this category) and a recent SCE study by Jostes et al. (1981) indicates that CHO cells do not show adaptive-DNA-repair with EMS.

In conclusion, the finding that MMS and X-ray combined treatments induce fewer SCEs in CHO cells than MMS-induced SCEs plus X-ray induced SCEs may be explained by a "cross reaction" of the MMS and X-ray repair machineries. The possibility that this effect might be due to an adaptive-DNA-repair system similar to the one described by Samson and Schwartz (1980) remains open to debate.

CHAPTER 4

SECTION 2: SCE INDUCTION BY ETHYL AND METHYL METHANESULFONATE

Introduction

In bacteria the repair of EMS and MMS induced DNA lesions appears to be governed by at least partly different repair pathways (Kondo et al., 1970). This contention is supported by the fact that EMS and MMS produce different spectra of alkylation lesions in DNA. For example, the ratio of O-6/N-7 alkylation of guanine is 0.004 and 0.03 for MMS and EMS respectively, the ratio of N-3 alkylation of adenine to N-7 alkylation of guanine is 0.12 for MMS and 0.08 for EMS (Lawley et al., 1975). One would therefore expect that the effects on SCE induction of these two agents should be additive. This hypothesis was tested in the following series of experiments. It is important to note that in the experiments presented in this Section and Sections 3 and 4 the chemicals were added simultaneously and hence the possibility of adaptive-DNA-repair effects on SCE may be excluded.

Materials and Methods

Asynchronously proliferating CHO cells were used in these experiments. For details of the culturing and harvesting procedures see the relevant Sections in Chapter 2. Sequential harvests were performed at 24, 26, and 28 hours for all but the last 2 experiments in this section (for these 2 only one harvest was done at 28 hours). Although second division cells appeared as early as 24 hours after the addition of low concentrations of mutagens to the cultures, a strong delay was induced in cultures treated with high mutagen concentrations and second division cells could not be obtained until the 28 hour harvest in those cultures. Therefore the 28 hour harvest time was selected for scoring

all cultures in all experiments. In all the experiments described below the mutagen(s) was added at the same time as BUdR (that is at time 0) and left in until the first harvest at 24 hours.

Experimental Design for EMS/MMS Experiments 1 to 6.

Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
Control	Control	Control	Control	Control	Control
EMS 30	MMS 10	MMS 10	MMS 10	MMS 10	MMS 10
EMS 20	MMS 5	MMS 5	MMS 5	MMS 5	MMS 5
EMS 10	MMS 4	MMS 4	MMS 4	MMS 4	MMS 4
EMS 3	MMS 3	MMS 3	MMS 3	MMS 3	MMS 3
	MMS 1	MMS 1	MMS 1	MMS 1	MMS 1
		EMS 20	EMS 10	EMS 5	EMS 3
		EMS 20 + MMS 10	EMS 10 + MMS 10	EMS 5 + MMS 10	EMS 3 + MMS 10
		EMS 20 + MMS 5	EMS 10 + MMS 5	EMS 5 + MMS 5	EMS 3 + MMS 5
		EMS 20 + MMS 4	EMS 10 + MMS 4	EMS 5 + MMS 4	EMS 3 + MMS 4
		EMS 20 + MMS 3	EMS 10 + MMS 3	EMS 5 + MMS 3	EMS 3 + MMS 3
		EMS 20 + MMS 1	EMS 10 + MMS 1	EMS 5 + MMS 1	EMS 3 + MMS 1

EMS values x 10^{-4}M; MMS values x 10^{-5}M

Results and Statistical Analysis

A regression line was calculated for MMS-induced SCEs by pooling MMS data from Experiments 2 to 6. The equation of the line is given below, where x is the MMS concentration multiplied by 10^5

$$\text{SCEs/cell} = 4.288x + 9.91$$

Ordinarily, one could have performed an ANOVAR treatment of the data to determine whether EMS plus MMS SCE values paralleled MMS values. However, this sort of statistical analysis requires that the variances of the means be very similar which was not the case for the data obtained. A statistical analysis of the data was devised with the assistance of Dr. Anthea Springbett.

If the effect of EMS and MMS on SCE induction is a strictly additive effect, one would expect that the points for EMS plus MMS to parallel the calculated MMS regression line with an upward shift in SCE frequency equivalent to the frequency of SCEs induced by EMS alone.

The 95% confidence limits of the estimated-expected value for each EMS plus MMS point was calculated by adding the 95% confidence interval of the regression line at any given point to the 95% confidence interval of the observed EMS plus MMS mean. This addition seemed justifiable since, as a rule, variance increases as SCE frequency increases.

Sample Calculation:

The 95% confidence interval for the MMS regression line at an MMS concentration of 5×10^{-5}M is:

$$\text{SCE/cell} = (4.288)(5) + 9.91 \pm [(2.048)(4.77)(\tfrac{1}{30} + \tfrac{5 - 3.83}{274})^{\frac{1}{2}}]$$

where 2.048 is the tabulated t value for n-2 degrees of freedom

4.77 is the estimated s for the regression line

30 is n, the number of means used to calculate the regression line

3.83 is $\bar{x}$ for the regression line

274 is S_{xx}

Therefore, SCE/cell = 31.35 ± 1.91

In experiment 4 the mean SCE frequency per cell was observed to be 54.95 for EMS 10 x 10^{-4}M + MMS 5 x 10^{-5}M. The standard error of the mean was 1.75. The 95% confidence interval for this mean is $54.95 \pm (2.093)(1.75)$, where 2.093 is the tabulated t value for 19 degrees of freedom (n=20 for each mean). The 95% confidence interval is therefore 54.95 ± 3.66.

The expected mean for EMS 10 x 10^{-4}M + MMS 5 x 10^{-5}M was calculated in the folowing way:

(EMS 10x10^{-4}M induced SCEs) - (base-line SCEs)

=33.40 - 9.91 = 24.21

24.21 + 31.35 = 55.56 = estimated expected mean SCE value for EMS 10x10^{-4}M + MMS 5x10^{-5}M

where 31.35 is the SCE value of the MMS regression line at x=5x10^{-5}M.

The 95% confidence limits for the expected SCE value are calculated by summing the values 3.66 and 1.91 to give:

55.56 ± 5.57, i.e. 49.99 to 61.13 expected SCEs.

The observed mean for EMS 10 x 10^{-4}M + MMS 5 x 10^{-5}M was 54.95 and falls within these limits. For this point it may be concluded that EMS and MMS SCE-inducing effects are additive.

Table 4.2.1. Mean SCE/Cell Induced by EMS ± Standard Error, S.E.
(Experiment 1, n = 20 cells/culture)

EMS Treatment (M)	SCE ± S.E.
Control	10.15 ± 0.65
30×10^{-4}	59.40 ± 3.14
20×10^{-4}	50.15 ± 1.79
10×10^{-4}	34.05 ± 1.47
3×10^{-4}	22.35 ± 2.02

Table 4.2.2. Mean SCE/Cell Induced by MMS ± Standard Error, S.E.
(Experiment 2, n = 20 cells/culture)

MMS Treatment (M)	SCE ± S.E.
Control	7.95 ± 0.70
10×10^{-5}	47.00 ± 1.92
5×10^{-5}	34.00 ± 1.64
4×10^{-5}	25.30 ± 1.40
3×10^{-5}	22.80 ± 0.99
1×10^{-5}	11.65 ± 0.66

Table 4 .2.3. Mean SCE/Cell ± Standard Error (S.E.) Induced by MMS (at various concentrations), EMS at 20 x 10^{-4}M and by Combined Treatment With EMS and MMS (Experiment 3, n = 20 cells).

MMS Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	20 x 10^{-4}	Expected SCE ± S.E.	ε
0	8.45 ± 0.61	51.60 ± 1.69		
10 x 10^{-5}	50.80 ± 2.35	76.20 ± 1.87	94.48 ± 7.96	<
5 x 10^{-5}	35.70 ± 1.70	61.85 ± 2.44	73.04 ± 7.07	<
4 x 10^{-5}	26.95 ± 1.19	59.65 ± 1.67	68.75 ± 4.69	<
3 x 10^{-5}	22.30 ± 1.37	61.30 ± 2.52	64.46 ± 6.17	=
1 x 10^{-5}	13.35 ± 0.62	50.80 ± 2.35	55.89 ± 6.11	=

column ε lists deviations from the expected:

< symbolises observed<expected

= symbolises observed=expected

> symbolises observed>expected

The expected SCE/cell and S.E. were calculated as described in pages 46 and 47.

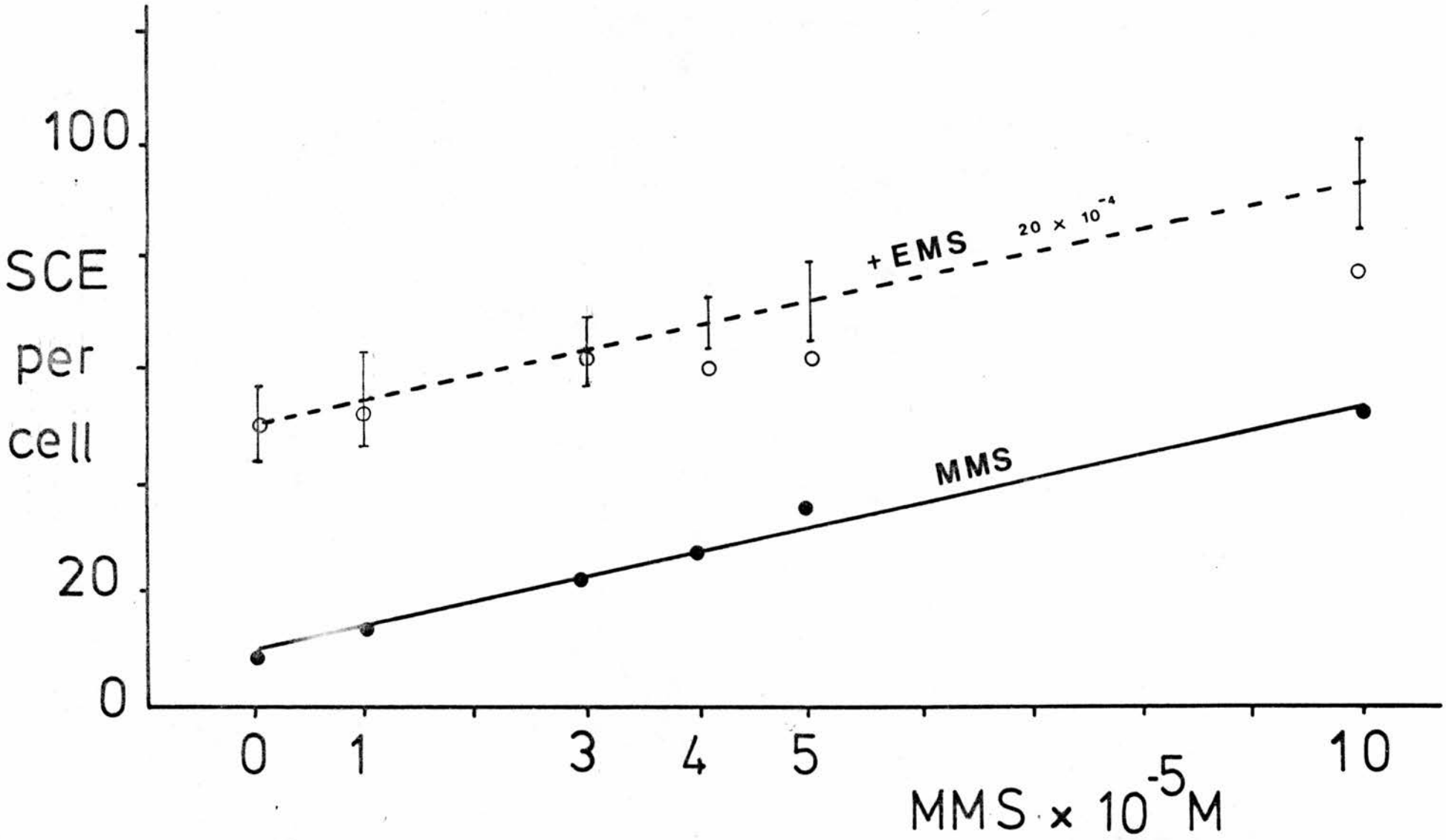

100
SCE
per
cell
20
0
+EMS 20 × 10⁻⁴
MMS
0
1
3
4
5
10
MMS × 10⁻⁵M

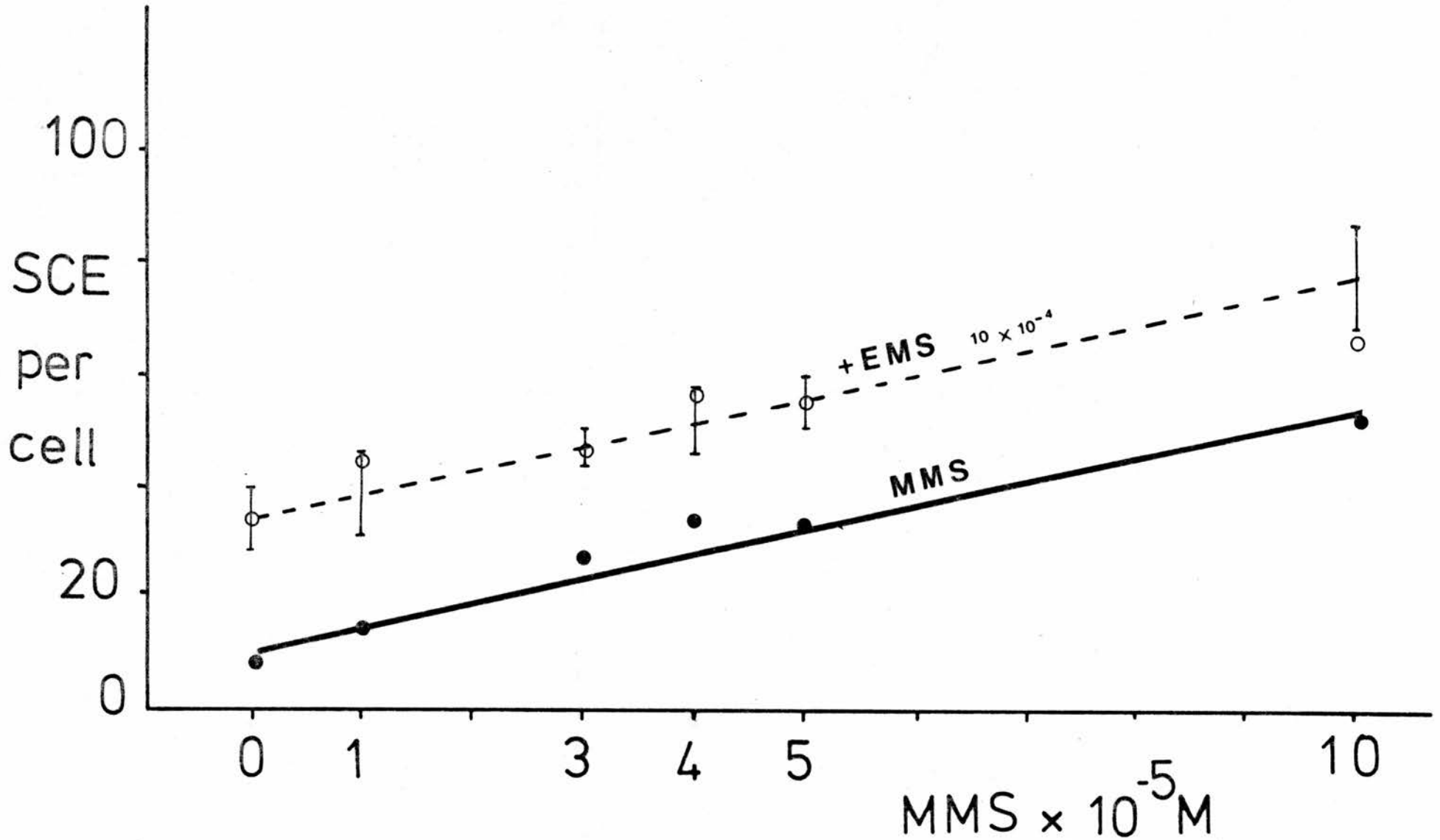
Table 4.2.4. Mean SCE/Cell ± Standard Error (S.E.) Induced by MMS (at various concentrations), EMS at 10×10^{-4}M and by Combined Treatment With EMS and MMS (Experiment 4, n = 20 cells).

MMS Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	10×10^{-4}	Expected SCE ± S.E.	ε
0	9.30 ± 0.79	33.40 ± 1.24		
10×10^{-5}	50.55 ± 1.57	65.70 ± 2.31	76.28 ± 8.88	<
5×10^{-5}	32.55 ± 1.35	54.95 ± 1.75	54.84 ± 5.58	=
4×10^{-5}	34.15 ± 1.37	55.70 ± 2.12	50.55 ± 5.63	=
3×10^{-5}	27.00 ± 1.11	45.40 ± 1.27	46.26 ± 3.56	=
1×10^{-5}	14.25 ± 0.90	43.50 ± 3.01	37.69 ± 7.49	=

Column description as in Table 4.2.3. (p. 49).

Table 4.2.5. Mean SCE/Cell ± Standard Error (S.E.) Induced by MMS (at various concentrations), EMS at 5×10^{-4}M and by Combined Treatment With EMS + MMS (Experiment 5, n = 20 cells).

MMS Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	5×10^{-4}	Expected SCE ± S.E.	ε
0	8.85 ± 0.50	26.40 ± 1.17		
10×10^{-5}	54.65 ± 1.86	Strong Delay,	no 2nd divisions	
5×10^{-5}	30.95 ± 1.16	47.95 ± 0.96	47.84 ± 3.92	=
4×10^{-5}	25.20 ± 0.79	47.35 ± 1.65	43.55 ± 4.64	=
3×10^{-5}	25.90 ± 1.16	36.70 ± 1.34	39.26 ± 3.70	=
1×10^{-5}	15.05 ± 0.63	31.85 ± 0.82	30.69 ± 2.91	=


100
SCE
per
cell
20
0
+EMS 10 × 10⁻⁴
MMS
0
1
3
4
5
10
MMS × 10⁻⁵M

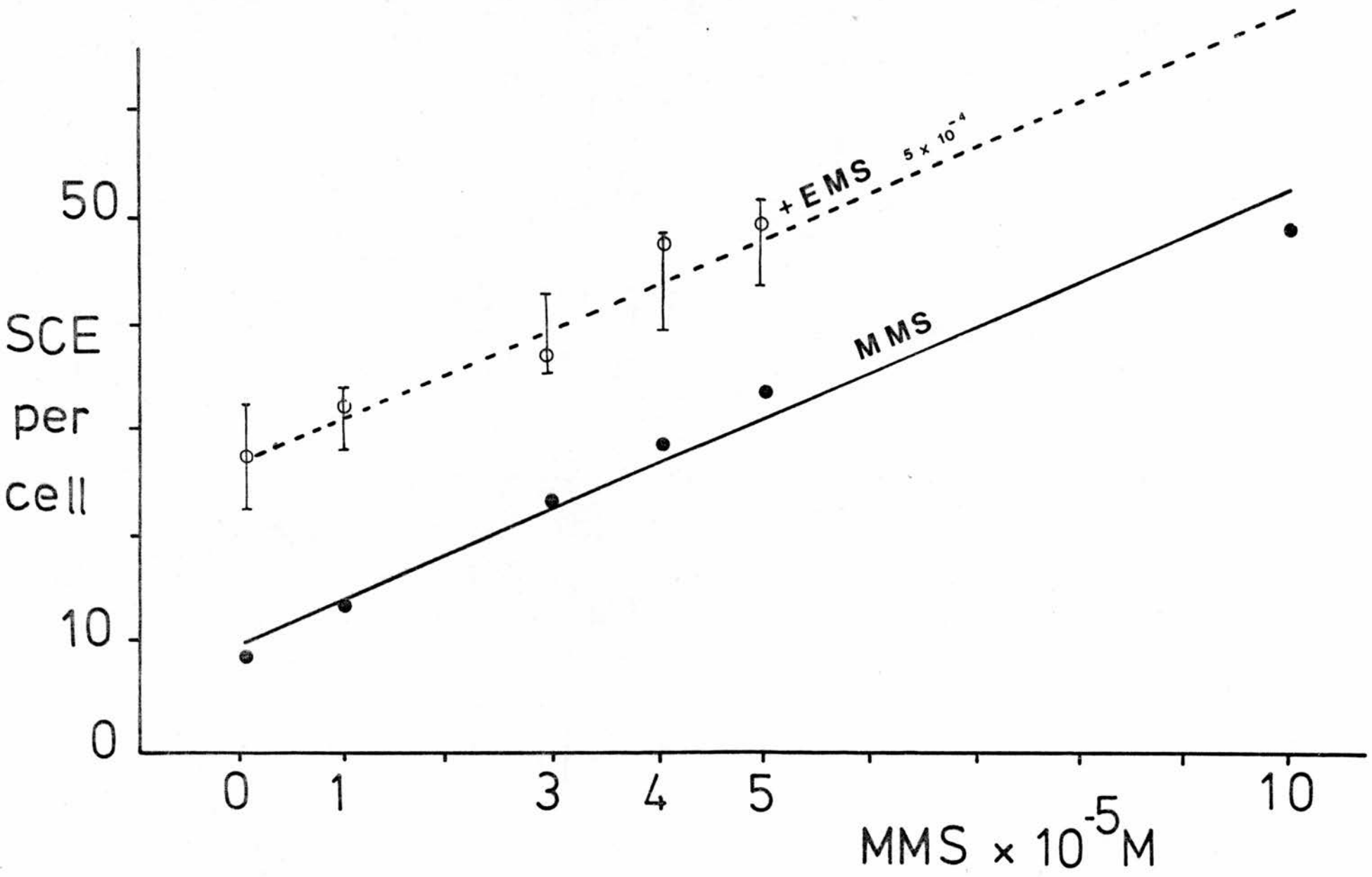
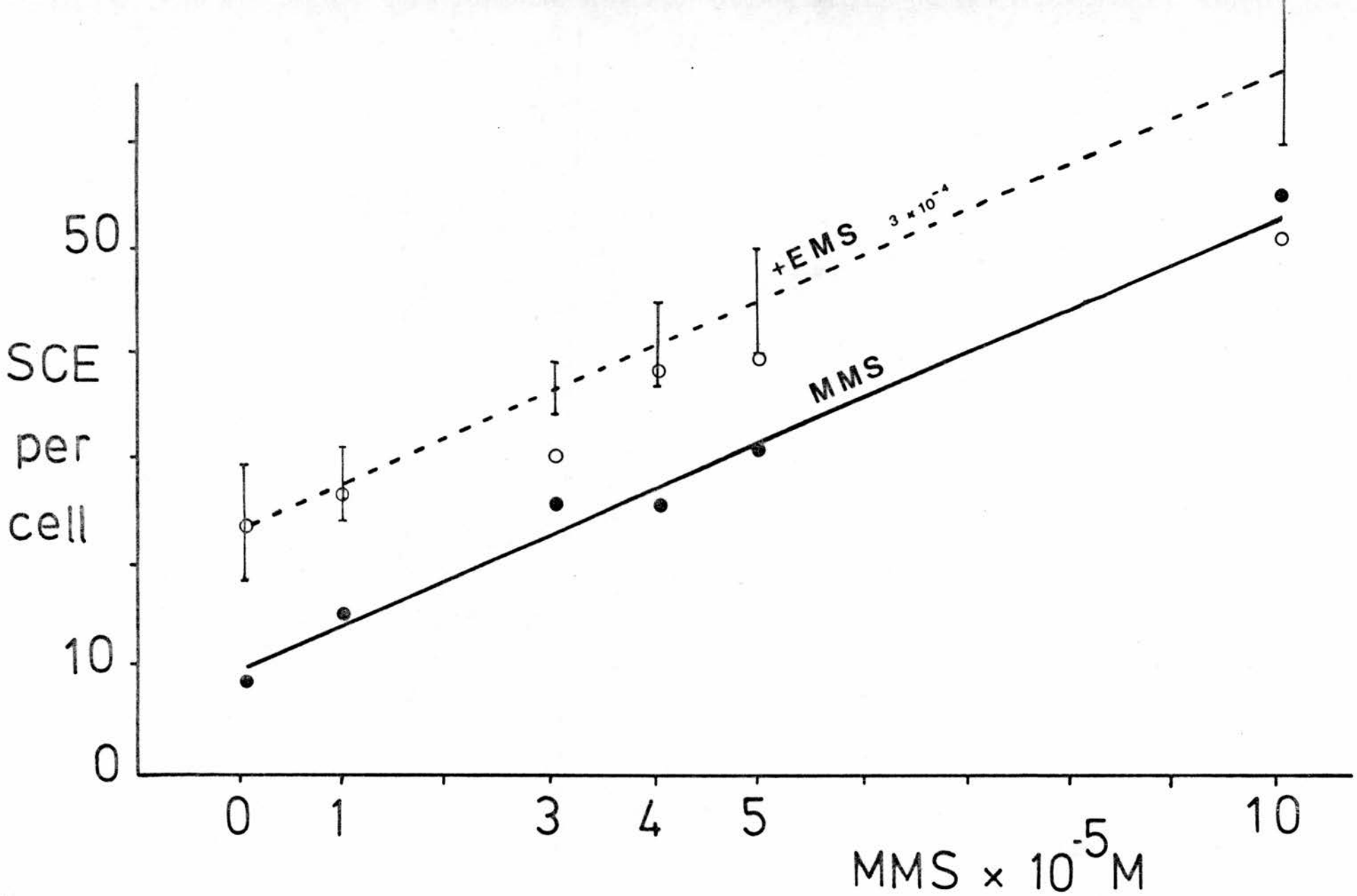

50
SCE
per
cell
10
0
+EMS 5 x 10⁻⁴
MMS
0
1
3
4
5
10
MMS x 10⁻⁵M

Table 4.2.6. Mean SCE/Cell ± Standard Error (S.E.) Induced by MMS (at various concentrations), EMS at 3×10^{-4}M and by Combined Treatment With EMS + MMS (Experiment 6, n = 20 cells).

MMS Treatment (M)	Observed SCE ± S.E. EMS Treatment (M)		Expected SCE ± S.E.	ε
	0	3×10^{-4}		
0	8.85 ± 0.50	23.35 ± 1.19		
10×10^{-5}	48.75 ± 1.79	50.75 ± 1.49	66.13 ± 7.17	<
5×10^{-5}	34.00 ± 1.33	39.15 ± 1.39	44.69 ± 4.82	<
4×10^{-5}	28.05 ± 1.14	37.70 ± 1.25	40.40 ± 3.81	=
3×10^{-5}	21.90 ± 1.19	30.00 ± 0.85	36.11 ± 2.68	<
1×10^{-5}	13.70 ± 0.99	26.15 ± 1.06	27.54 ± 3.41	=

Column description as in Table 4.2.3. (p. 49).


50
SCE
per
cell
10
0
+EMS 3 x 10⁻⁴
MMS
0
1
3
4
5
10
MMS x 10⁻⁵M

Preliminary Discussion of EMS and MMS Results

The data presented in Section 2 of this chapter indicate, with regard to the issue of involvement of O-6-alkylG, that O-6-alkylG cannot be the only lesion responsible for SCE induction since MMS causes only a negligible quantity of O-6-alkylG relative to EMS (Lawley et al., 1975) but is much more efficient at inducing SCEs on a molar basis than EMS.

EMS and MMS combined treatments induced as many SCEs as (EMS-alone-induced SCEs) + (MMS-alone-induced SCEs) within the expected limits for SCE induction for combined treatment. This result is consistent with the idea that EMS and MMS repair occurs via at least partly different pathways (Kondo et al., 1970). At high concentrations of EMS and MMS, combined treatment resulted in fewer SCEs than expected (Tables 4.2.3. and 4.2.4.). This may be explained by a "saturation effect" on SCE induction, that is at high mutagen concentrations the SCE frequency begins to level off and the cytotoxic effects of the mutagen begin to interfere in the system to such an extent that second division metaphases cannot be obtained. In this connection it is interesting to note that EMS and MMS begin to "saturate" the SCE frequency at about 50-60 SCEs/cell and that combined treatment of EMS and MMS follows the same trend. The significance of this observation is unclear.

Why three out of five (one of these three is only marginally lower than the expected) combined treatments of EMS at 3×10^{-4}M plus MMS should be lower than the expected remains unclear (see Table 4.2.6). This may well be a spurious result because all the SCE frequency values obtained for combined EMS and MMS treatment in Experiment 5 (in which EMS at 5×10^{-4}M induced on average only about 3 more SCEs per cell than an EMS concentration of 3×10^{-4}M) fell within the expected limits.

CHAPTER 4

SECTION 3: SCE INDUCTION BY ETHYL METHANESULFONATE AND ETHYL NITROSOUREA

Introduction

Treatment of CHO cells with single and combined doses of EMS and ENU was investigated in this Section. ENU produces 25 times more O-6-ethylG than EMS (Sun and Singer, 1975). If alkylation of the O-6 position of guanine is important in SCE induction, one might expect ENU to be more efficient, on a molar basis, than EMS at inducing SCEs. Futhermore, combined treatment with EMS and ENU should be additive in terms of SCE induction since the alkylation spectra for these mutagens are so different (for example, 8% of EMS alkylation products are ethyl-phosphates whereas 66% of ENU alkylation products are ethyl-phosphates, Sun and Singer, 1975).

Materials and Methods

As for the experiments in Section 2 of this Chapter. asynchronously dividing CHO cells were exposed for 24 hours (two cell cycles in the presence of BUdR) to various concentrations of EMS and ENU. The 28 hour harvests were selected for scoring. Details of the exposure protocols are listed on the following page.

Experimental Design for EMS/ENU Experiment 1 and 2

Exp. 1	Exp. 2	
	No DMSO	
DMSO 0.4%	DMSO 0.4%	
ENU 10	ENU 5 + EMS 20	ENU 4 + EMS 20
ENU 5	ENU 5 + EMS 10	ENU 4 + EMS 10
ENU 4	ENU 5 + EMS 5	ENU 4 + EMS 5
ENU 3	ENU 5 + EMS 3	ENU 4 + EMS 3
ENU 2	ENU 5	ENU 4
	EMS 20	
	EMS 10	
	EMS 5	
	EMS 3	

EMS concentrations x 10^{-4}M, ENU concentrations x 10^{-4}M

Results and Statistical Analysis

The data obtained in these two experiments were statistically analysed as described for the experiments of Section 2 of this Chapter (see pages 46 and 47).

A regression line was calculated for the SCE/cell induced by various doses of EMS. The equation of the line is given below, where x is the EMS concentration x 10^4

$$SCEs/cell = 2.62x + 12.88$$

Table 4.3.1. Mean SCE/cell ± Standard Error (S.E.)
Induced by ENU Alone (Experiment 1, n=20 cells)

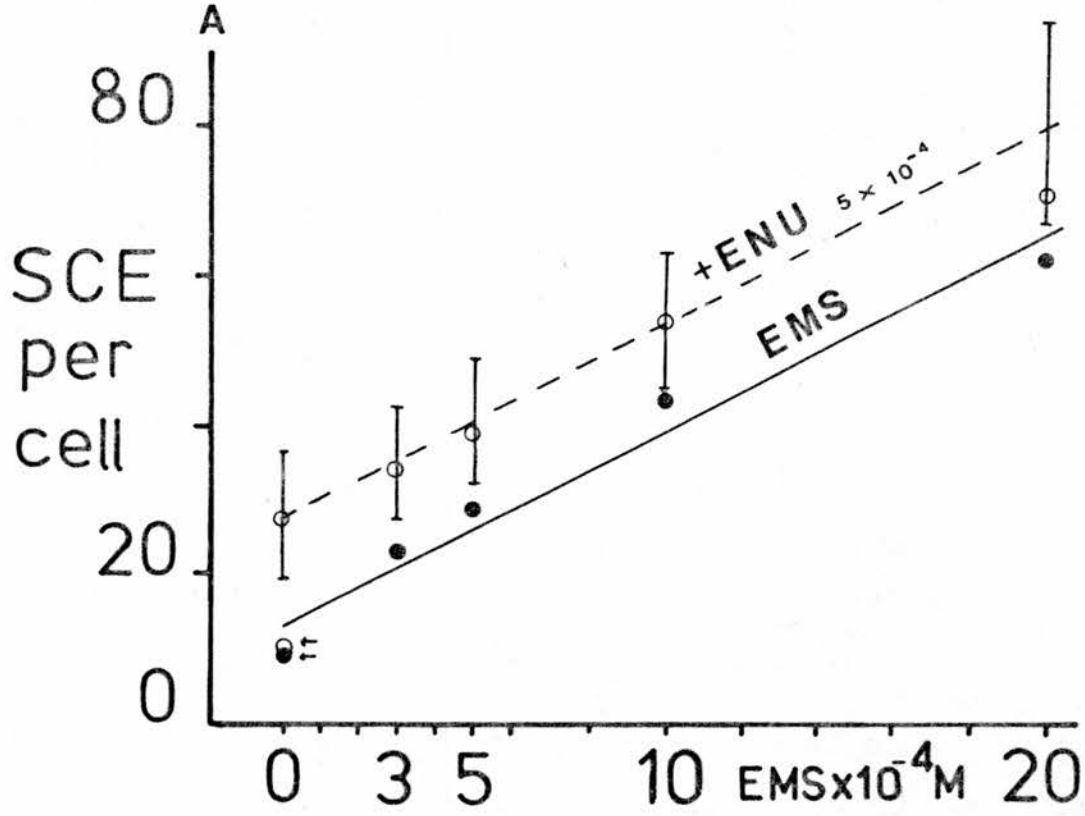
ENU Treatment (M)	SCE ± S.E.
DMSO 0.4%	9.60 ± 0.52
10×10^{-4}	toxic
5×10^{-4}	29.30 ± 1.09
4×10^{-4}	25.30 ± 1.41
3×10^{-4}	18.65 ± 1.63
2×10^{-4}	18.30 ± 1.70

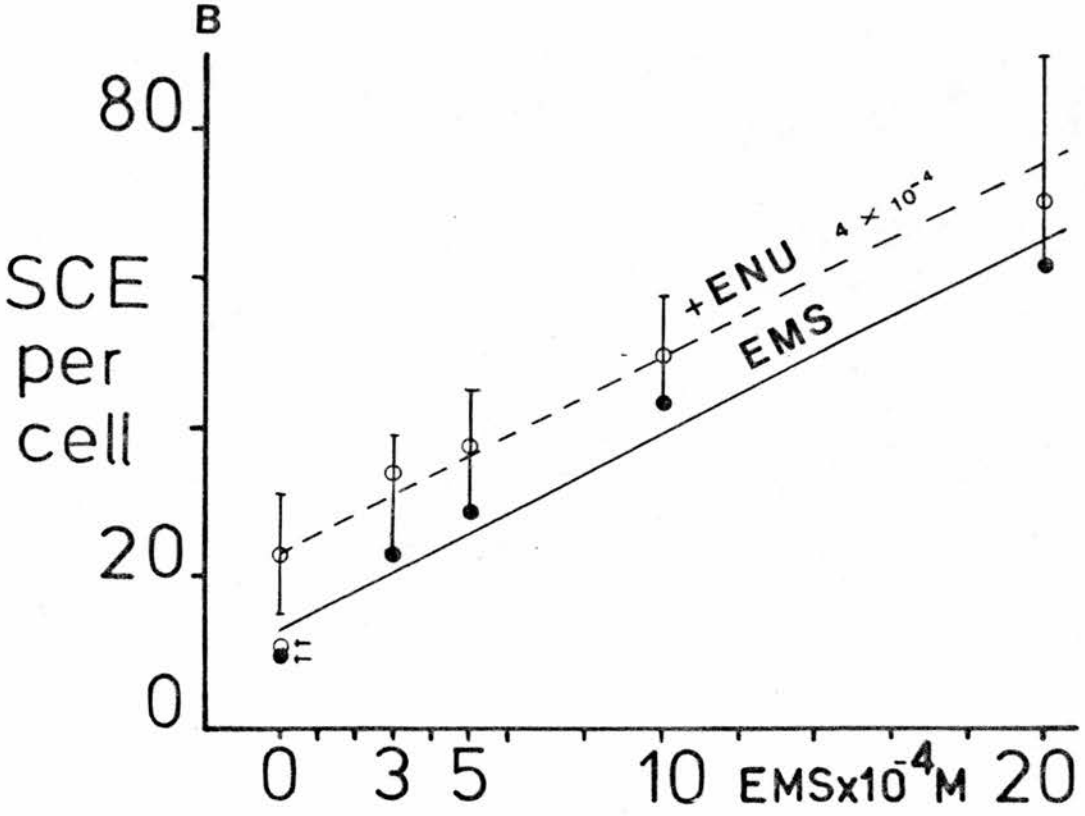
Table 4.3.2. Mean SCE/Cell ± Standard Error (S.E.) Induced by EMS (at various concentrations), ENU at 5×10^{-4}M and 4×10^{-4}M, and by Combined Treatment With EMS and ENU (Experiment 2, n = 20 cells).

EMS Treatment (M)	Observed SCE ± S.E. ENU Treatment (M) 0	5×10^{-4}	Expected SCE/cell ± S.E.	ε	Observed SCE ± S.E. 4×10^{-4}	Expected SCE/cell ± S.E.	ε
0	9.75 ± 0.58						
DMSO 0.4%	10.10 ± 1.11	28.10 ± 1.22			23.10 ± 1.12		
20×10^{-4}	61.90 ± 1.65	75.50 ± 1.64	81.25 ±13.83	=	69.90 ± 1.81	75.50 ±14.19	=
10×10^{-4}	43.65 ± 1.78	53.85 ± 1.80	55.05 ± 9.12	=	49.55 ± 1.58	49.30 ± 8.66	=
5×10^{-4}	28.85 ± 1.33	38.90 ± 1.48	41.95 ± 7.92	=	37.60 ± 1.47	36.20 ± 7.90	=
3×10^{-4}	23.35 ± 1.12	34.55 ± 2.34	36.71 ± 7.59	=	34.25 ± 1.52	30.96 ± 8.43	=

Column ε lists deviations from the expected: No deviations from the expected were observed in this experiment (symbolised by = in the ε column).

The expected SCE/cell and S.E. were calculated as described in pages 46 and 47.


A
80
SCE
per
cell
20
0
+ENU 5 × 10⁻⁴
EMS
0 3 5 10 EMSx10⁻⁴M 20


B
80
SCE
per
cell
20
0
+ENU 4 × 10⁻⁴
EMS
0 3 5 10 EMSx10⁻⁴M 20

Preliminary Discussion for EMS and ENU Results

The results obtained in this Section again do not support the notion that O-6-alkylG is the only lesion involved in the generation of SCE since equimolar concentrations of EMS and ENU (4×10^{-4}M) produced approximately the same SCE frequency (23 and 28 SCEs/cell for EMS and ENU, respectively) but ENU causes 25 times more ethylation at the O-6 site of guanine than EMS (Sun and Singer, 1975).

The combined treatment (EMS + ENU) effect on SCE induction was additive within the expected limits. This result is in agreement with the fact that EMS and ENU produce such different spectra of DNA lesions.

CHAPTER 4

SECTION 4: SCE INDUCTION BY ANTHRAMYCIN AND ETHYL METHANESULFONATE, AND ETHYL NITROSOUREA

Introduction

Anthramycin (ANT) is an infrequently studied anti-tumor agent. For this reason a more extensive description of the effects of ANT will be given. ANT has a much higher affinity for double stranded DNA than for single stranded DNA (Hurley et al., 1977, Kohn and Spears, 1970) and binds neither to RNA nor to protein (Hurley et al., 1977). The ANT-DNA conjugate is extremely stable (Hurley et al., 1979b). The notion that ANT binding to DNA might be highly specific was introduced by Stefanovic (1968) and it was later shown that ANT has a high affinity for guanine (Hurley, 1977, Kohn et al., 1968, Kohn and Spears, 1970, Kohn et al., 1974). In 1979 Hurley and Petrusek reported that ANT covalently binds to DNA at the N-2 position of guanine exclusively. Glaubiger (1974) had found that ANT does not distort or extend the DNA helix but rather that it stiffens it (but does not intercalate). Hurley and Petrusek's finding that ANT covalently binds to the N-2 position of guanine and snugly fits (without distortion or protrusion) into the narrow groove of the helix explains the slow removal of ANT from DNA observed by Hurley et al. (1979a) in human fibroblasts.

It was therefore of interest to see whether ANT could induce SCEs and, if so, whether ANT + EMS and ANT + ENU combined treatments would be additive with respect to SCE induction. No N-2 ethylation of DNA has been reported for EMS at neutral pH, though some N-2 ethylation of guanine has been reported by Lawley et al. (1975) at pH 12-13. No ethylation of the N-2 position of guanine has been reported for ENU.

Material and Methods

Asynchronously dividing CHO cells were exposed to ANT for 24 hours (2 cell cycles in BUdR). Since ANT was found to be positive for SCE induction, combined treatments of ANT + EMS and ANT + ENU were investigated. The 28 hour harvests were selected for scoring.

Experimental Design for ANT/ EMS and ANT/ENU Experiments 1 to 6.

Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
DMSO .5%	DMSO .5%	DMSO .5%	No DMSO DMSO .5%	No DMSO DMSO .5%	No DMSO DMSO .9%
ANT 64 ANT 32 ANT 16 ANT 8 ANT 4	ANT 64 ANT 32 ANT 16 ANT 8	ANT 64 ANT 32 ANT 16 ANT 8	ANT 32 ANT 16 ANT 8 ANT 4	ANT 32 ANT 16 ANT 8 ANT 4	ANT 64 ANT 32 ANT 16 ANT 8
	EMS 20	EMS 10	EMS 5	EMS 3	ENU 4
	EMS 20 + ANT 64	EMS 10 + ANT 64	EMS 5 + ANT 32	EMS 3 + ANT 32	ENU 4 + ANT 64
	EMS 20 + ANT 32	EMS 10 + ANT 32	EMS 5 + ANT 16	EMS 3 + ANT 16	ENU 4 + ANT 32
	EMS 20 + ANT 16	EMS 10 + ANT 16	EMS 5 + ANT 8	EMS 3 + ANT 8	ENU 4 + ANT 16
	EMS 20 + ANT 8	EMS 10 + ANT 8	EMS 5 + ANT 4	EMS 3 + ANT 4	ENU 4 + ANT 8

ANT values x 10^{-9}M, EMS x 10^{-4}M, ENU x 10^{-4}M

Results and Statistical analysis

The data obtained in the experiments described in this Section were statistically analysed as described for the experiments in Section 2 of this Chapter (see pages 46 and 47).

A regression line was calculated for the SCE/cell induced by various concentrations of ANT. The equation of the line is given below, where x = ANT concentration x 10^9

SCEs/cell = 0.7087x + 10.83

Table 4.4.1. Mean SCE/Cell ± Standard Error (S.E.) Induced by ANT (Experiment 1, n = 20 cells).

ANT Treatment (M)	SCE ± S.E.
DMSO 0.5%	8.60 ± 0.67
64 x 10^{-9}	53.00 ± 2.10
32 x 10^{-9}	38.40 ± 1.77
16 x 10^{-9}	25.00 ± 1.22
8 x 10^{-9}	17.40 ± 1.15
4 x 10^{-9}	12.45 ± 0.75

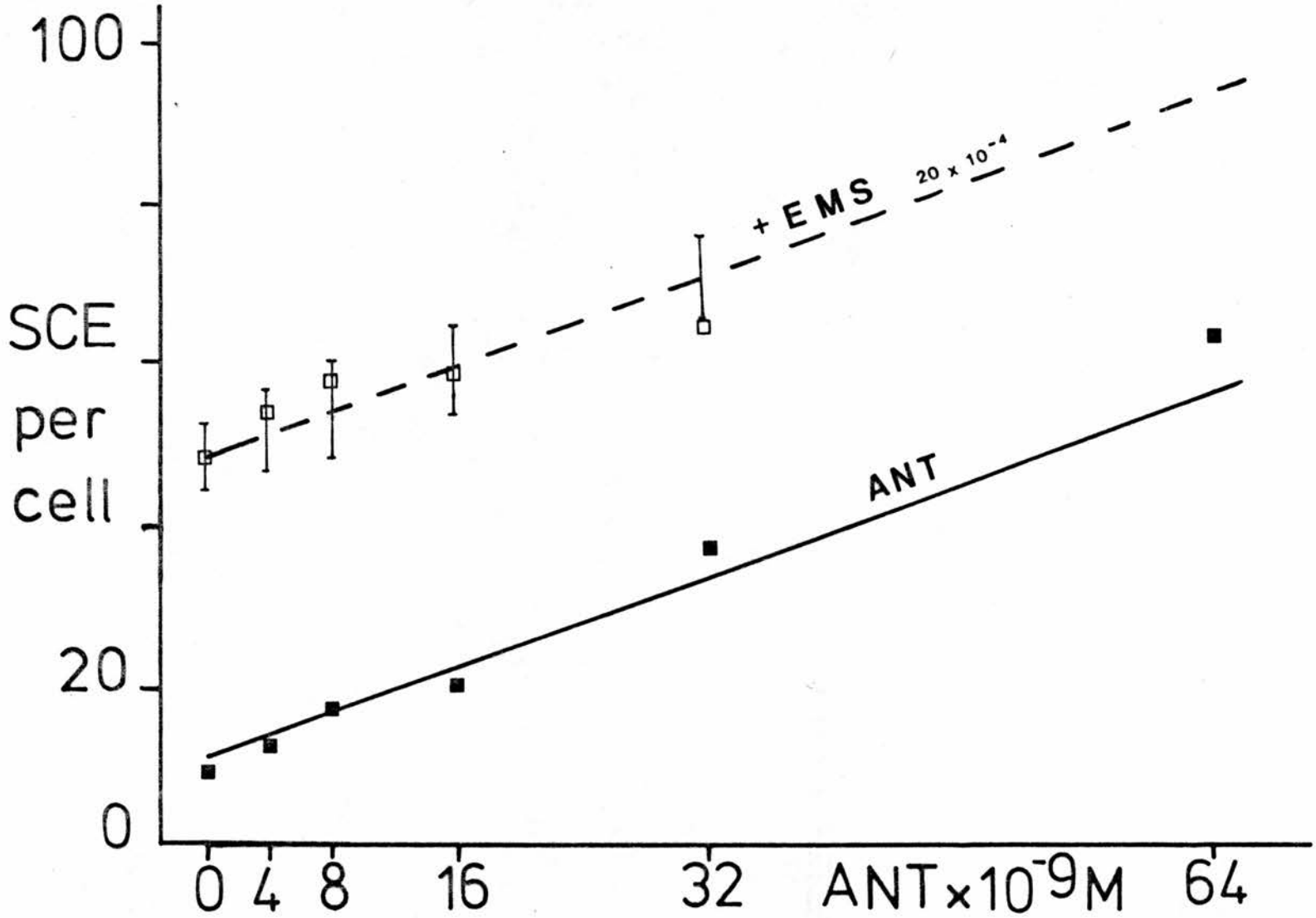
Table 4.4.2. Mean SCE/Cell ± Standard Error (S.E.) Induced by ANT (at various concentrations), EMS at 20×10^{-4}M and by Combined Treatment ANT + EMS (Experiment 2, n = 20 cells).

ANT Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	20×10^{-4}	Expected SCE ± S.E.	ε
DMSO 0.5%	9.15 ± 0.52	48.05 ± 1.08		
64×10^{-9}	62.90 ± 2.79	Strong Delay, no 2nd divisions		
32×10^{-9}	37.10 ± 2.23	63.90 ± 1.84	70.52 ± 5.34	<
16×10^{-9}	19.95 ± 1.00	59.00 ± 2.12	59.29 ± 5.70	=
8×10^{-9}	16.70 ± 0.77	58.15 ± 2.09	53.67 ± 5.78	=
4×10^{-9}	12.45 ± 0.77	54.00 ± 2.63	50.86 ± 6.34	=

Column ε lists deviations from the expected:
< symbolises observed < expected; = symbolises observed=expected

Table 4.4.3. Mean SCE/Cell ± Standard Error (S.E.) Induced by ANT (at various concentrations), EMS at 10×10^{-4}M and by Combined Treatment ANT + EMS (Experiment 3, n = 20 cells).

ANT Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	10×10^{-4}	Expected SCE ± S.E.	ε
DMSO 0.5%	9.65 ± 0.73	40.75 ± 1.91		
64×10^{-9}	50.65 ± 2.05	Strong delay, no 2nd divisions		
32×10^{-9}	36.60 ± 1.60	56.95 ± 1.47	63.22 ± 4.57	<
16×10^{-9}	24.95 ± 1.18	46.90 ± 1.47	51.99 ± 4.34	<
8×10^{-9}	14.90 ± 0.62	45.65 ± 1.38	46.37 ± 4.30	=


100
SCE
per
cell
20
0
+ EMS 20 x 10⁻⁴
ANT
0 4 8 16 32 ANT x 10⁻⁹M 64

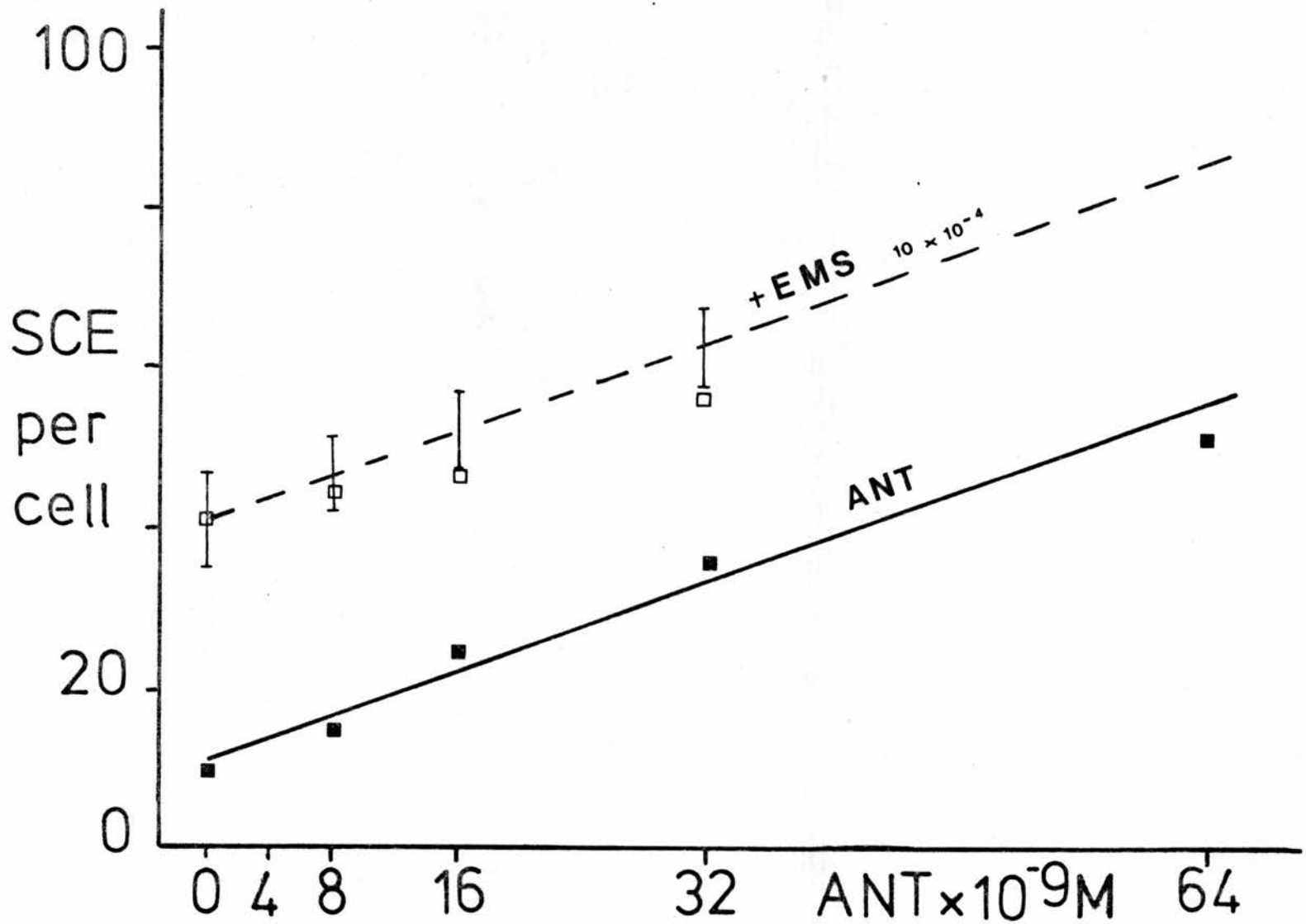

100
SCE
per
cell
20
0
+EMS 10 × 10⁻⁴
ANT
0 4 8 16 32 ANT×10⁻⁹M 64

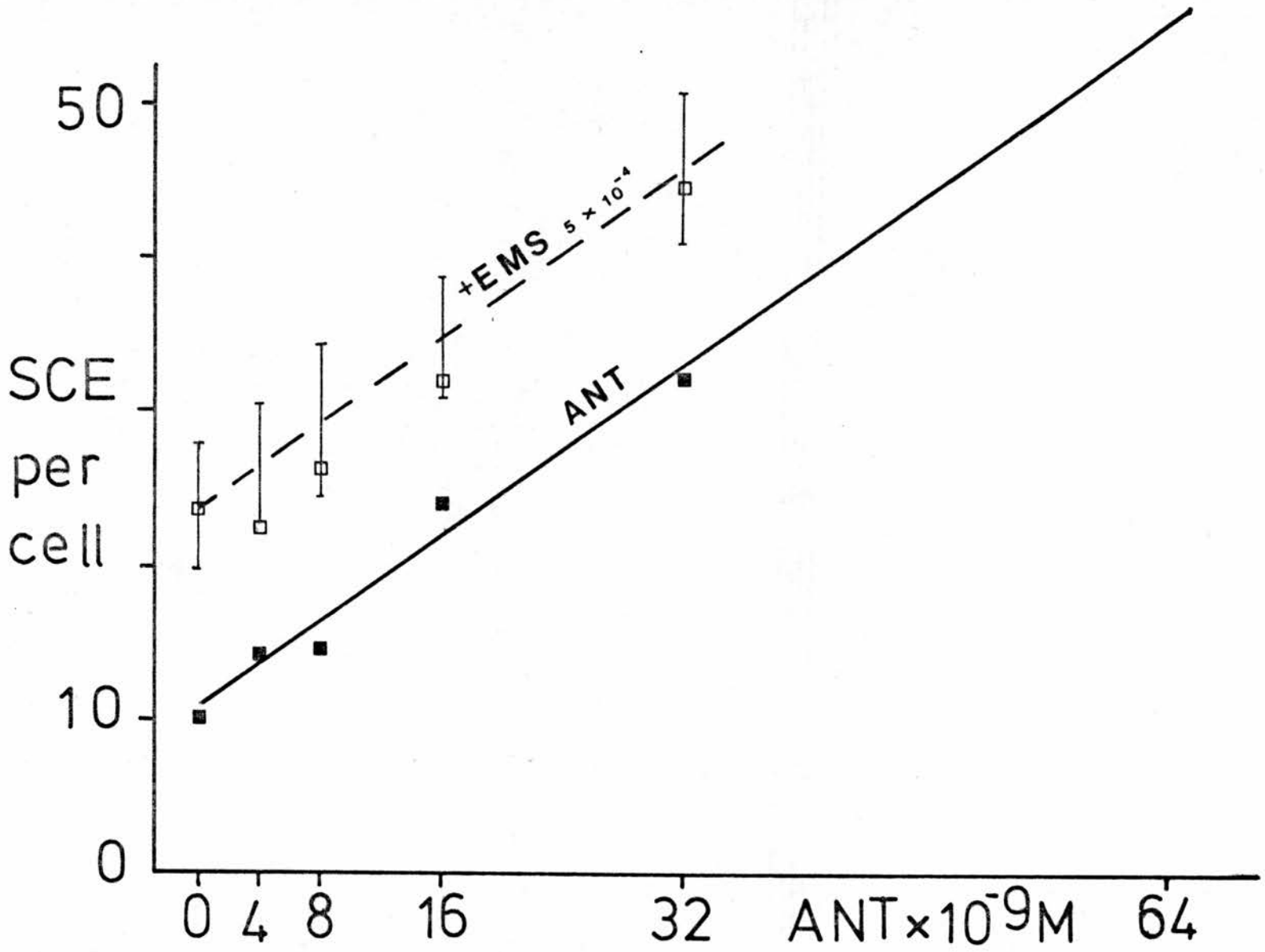
Table 4.4.4. Mean SCE/Cell ± Standard Error (S.E.) Induced by ANT (at various concentrations), EMS 5×10^{-4}M and by Combined Treatment ANT + EMS (Experiment 4, n = 20 cells).

ANT Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	5×10^{-4}	Expected SCE ± S.E.	ε
No DMSO	9.40 ± 0.54	23.75 ± 1.06		
DMSO 0.5%	10.05 ± 0.73			
32×10^{-9}	31.90 ± 1.87	44.35 ± 1.62	46.22 ± 4.88	=
16×10^{-9}	23.80 ± 1.21	31.65 ± 1.33	34.99 ± 4.04	=
8×10^{-9}	14.50 ± 0.84	26.20 ± 1.76	29.37 ± 5.09	=
4×10^{-9}	14.00 ± 0.77	22.25 ± 1.11	26.56 ± 3.85	<

< in the ε column symbolises observed < expected
= in the ε column symbolises observed = expected

Table 4.4.5. Mean SCE/Cell ± Standard Error (S.E.) Induced by ANT (at various concentrations), EMS at 3×10^{-4}M and by Combined Treatment ANT + EMS (Experiment 5, n = 20 cells).

ANT Treatment (M)	Observed SCE ± S.E. EMS Treatment (M) 0	3×10^{-4}	Expected SCE ± S.E.	ε
No DMSO	9.20 ± 0.67	19.45 ± 0.81		
DMSO 0.5%	9.65 ± 0.63			
32×10^{-9}	35.95 ± 1.74	41.55 ± 1.40	41.92 ± 4.42	=
16×10^{-9}	24.70 ± 1.31	29.75 ± 1.20	30.69 ± 3.77	=
8×10^{-9}	14.80 ± 0.90	22.20 ± 1.16	25.07 ± 3.83	=
4×10^{-9}	13.40 ± 1.02	19.95 ± 1.13	22.26 ± 3.90	=


50
SCE
per
cell
10
0
+EMS 5 × 10⁻⁴
ANT
0 4 8 16 32 ANT×10⁻⁹M 64

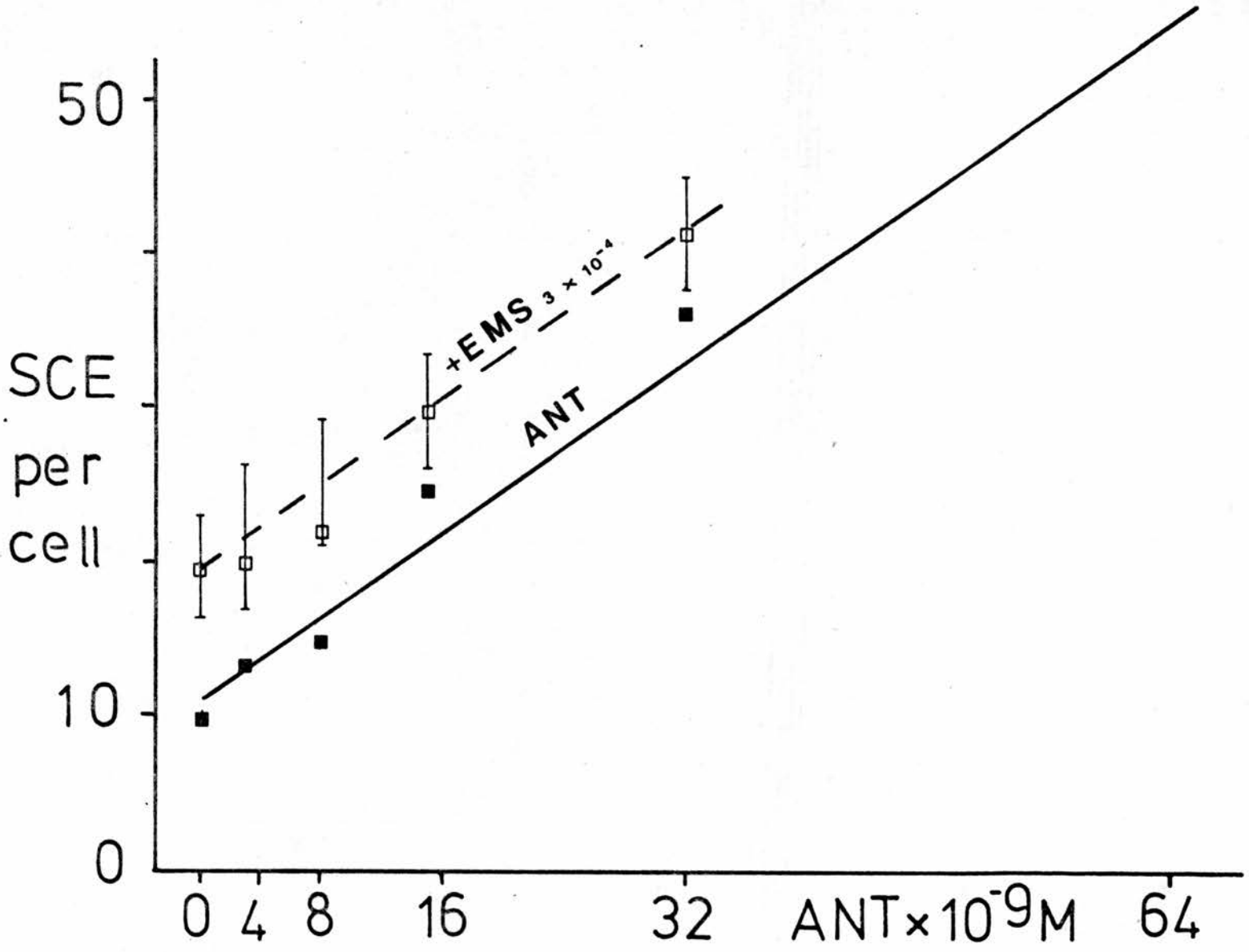
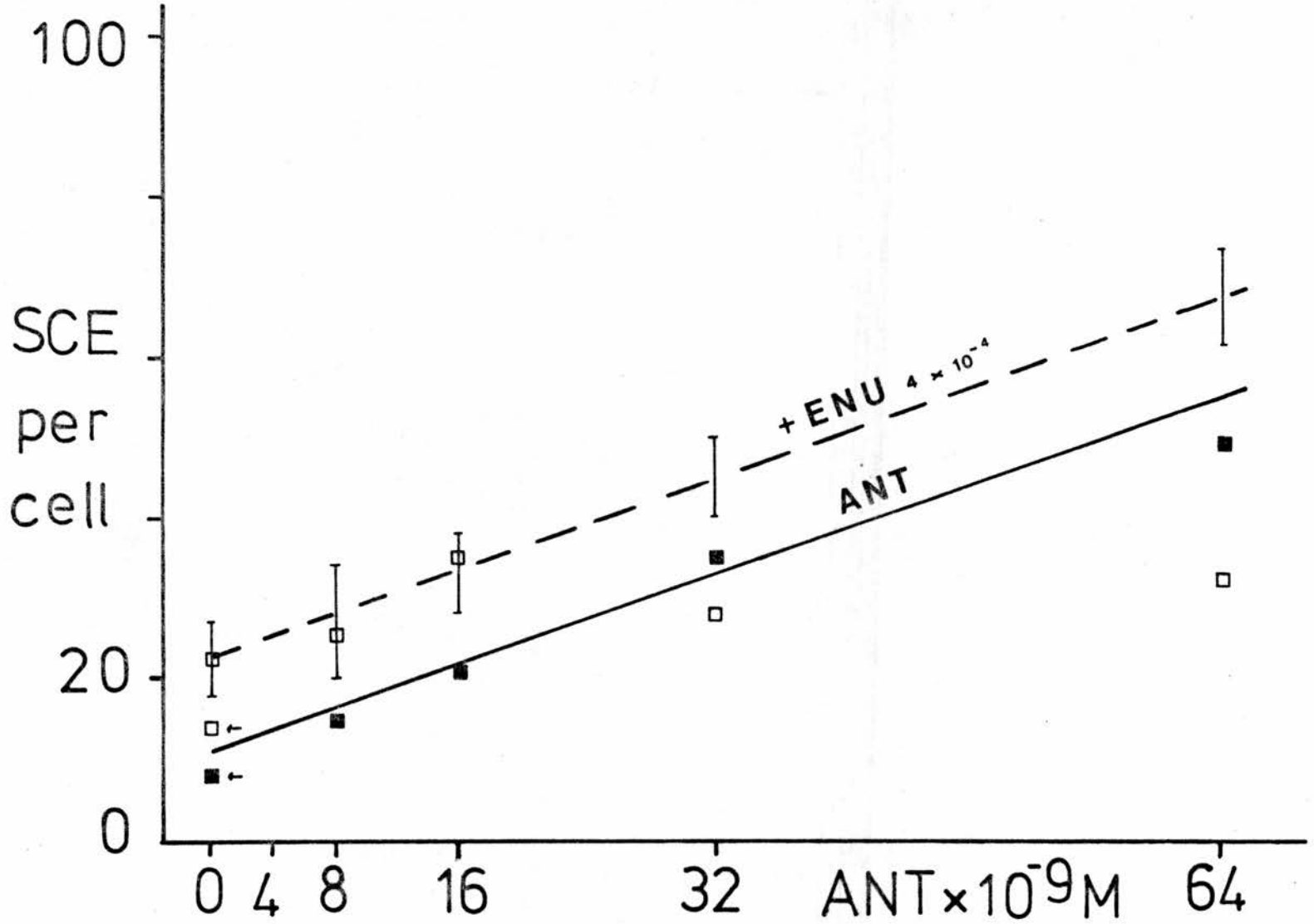

50
SCE
per
cell
10
0
+EMS 3 × 10⁻⁴
ANT
0 4 8 16 32 ANT×10⁻⁹M 64

Table 4.4.6. Mean SCE/Cell $\pm$ Standard Error (S.E.) Induced by ANT (at various concentrations), ENU at 4×10^{-4}M and by Combined Treatment ANT + ENU (Experiment 6, n = 20 cells).

ANT Treatment (M)	Observed SCE $\pm$ S,E. ENU Treatment (M)		Expected SCE $\pm$ S.E.	
	DMSO 0.4%	4×10^{-4}		ε
No DMSO	8.95 $\pm$ 0.67	22.70 $\pm$ 1.45		
DMSO 0.5%	13.85 $\pm$ 0.96			
64×10^{-9}	50.55 $\pm$ 1.95	30.45 $\pm$ 1.39	67.65 $\pm$ 5.95	<
32×10^{-9}	36.60 $\pm$ 1.60	28.05 $\pm$ 1.86	45.17 $\pm$ 5.38	<
16×10^{-9}	22.15 $\pm$ 0.87	35.55 $\pm$ 1.71	33.94 $\pm$ 4.84	=
8×10^{-9}	15.65 $\pm$ 1.04	25.70 $\pm$ 2.11	28.32 $\pm$ 5.82	=

< in ε column symbolises observed < expected

= in ε column symbolises observed = expected


100
SCE
per
cell
20
0
+ ENU 4 × 10⁻⁴
ANT
0 4 8 16 32 ANT×10⁻⁹M 64

Preliminary Dicussion of ANT/EMS Results

It was found that ANT induces a substantial number of SCEs at very low concentrations of the chemical (Table 4.4.1). While these experiments were in progress Brat and Dosik (1979) reported that ANT induces SCEs in chromosomes of Indian muntjac. Their paper was presumably in press when Hurley and Petrusek (1979) reported that ANT binds exclusively to the N-2 position of guanine and therefore Brat and Dosik were unable to make the obvious conclusion that O-6-alkylG is not the only lesion involved in the generation of SCE. The fact that ANT induces approximately 50 SCEs/cell at a concentration of 64×10^{-9}M clearly supports this conclusion. EMS, which does alkylate the O-6 position of guanine, must be administered at a concentration of 20×10^{-4}M to induce 50 SCEs/cell.

ANT plus EMS combined treatment was generally additive with respect to SCE induction although some SCE values were marginally lower than the expected values. This observation corroborates the notion that ANT and EMS do not compete for "SCE sites".

ANT/ENU Discussion

A detailed discussion of ANT and ENU interactions will be given at the end of Section 5. The rather spectacular discrepancy between the observed and the expected SCE frequencies following combined ANT + ENU treatment led to re-investigation of the experiment (Experiment 6). Section 5 of this Chapter deals with further experiments involving combined treatment of CHO cells with ANT + ENU.

CHAPTER 4

SECTION 5 SCE INDUCTION BY ANTHRAMYCIN AND ETHYL NITROSOUREA, FURTHER INVESTIGATIONS.

Introduction

As was mentioned in the foregoing page, the very large discrepancy between the expected and the observed SCE frequencies following ANT + ENU combined treatment warranted further experiments (results of ANT + ENU are given in Section 4, Table 4.4.6. and Figure 4.4.5.). Since the effect of an EMS dose which induces approximately 20 SCEs/cell plus any ANT dose was additive with respect to SCE induction (Table 4.4.5.), it did not seem unreasonable to speculate that some kind of interactive inhibition had occurred between ANT and ENU. To test this conjecture the experiment was repeated but with a "split dose" treatment in order to eliminate any extra-cellular ANT-ENU interactions.

Results and Statistical Analysis of the Data

The results of ANT + ENU split dose experiments 1 to 4 are listed in Tables 4.5.1. to 4.5.4., adjacent to each treatment protocol.

A t-test was applied to the data. The expected SCE frequency for ANT + ENU treatment was calculated by pooling ANT- and ENU-induced SCEs and correcting for (i.e. subtracting) the base-line SCE frequencies (pooled DMSO and DMSO-free controls). The results of the statistical analysis are presented in Table 4.5.5.

Table 4.5.1. ANT + ENU Induced SCEs ± Standard Error.

ANT and ENU were given either together during the 1st hour of culture or as split doses separated by 2 hours in mutagen-free conditions. Cells were harvested at 28 hours.

ANT concentration: 25×10^{-7}M; ENU concentration: 5×10^{-4}M

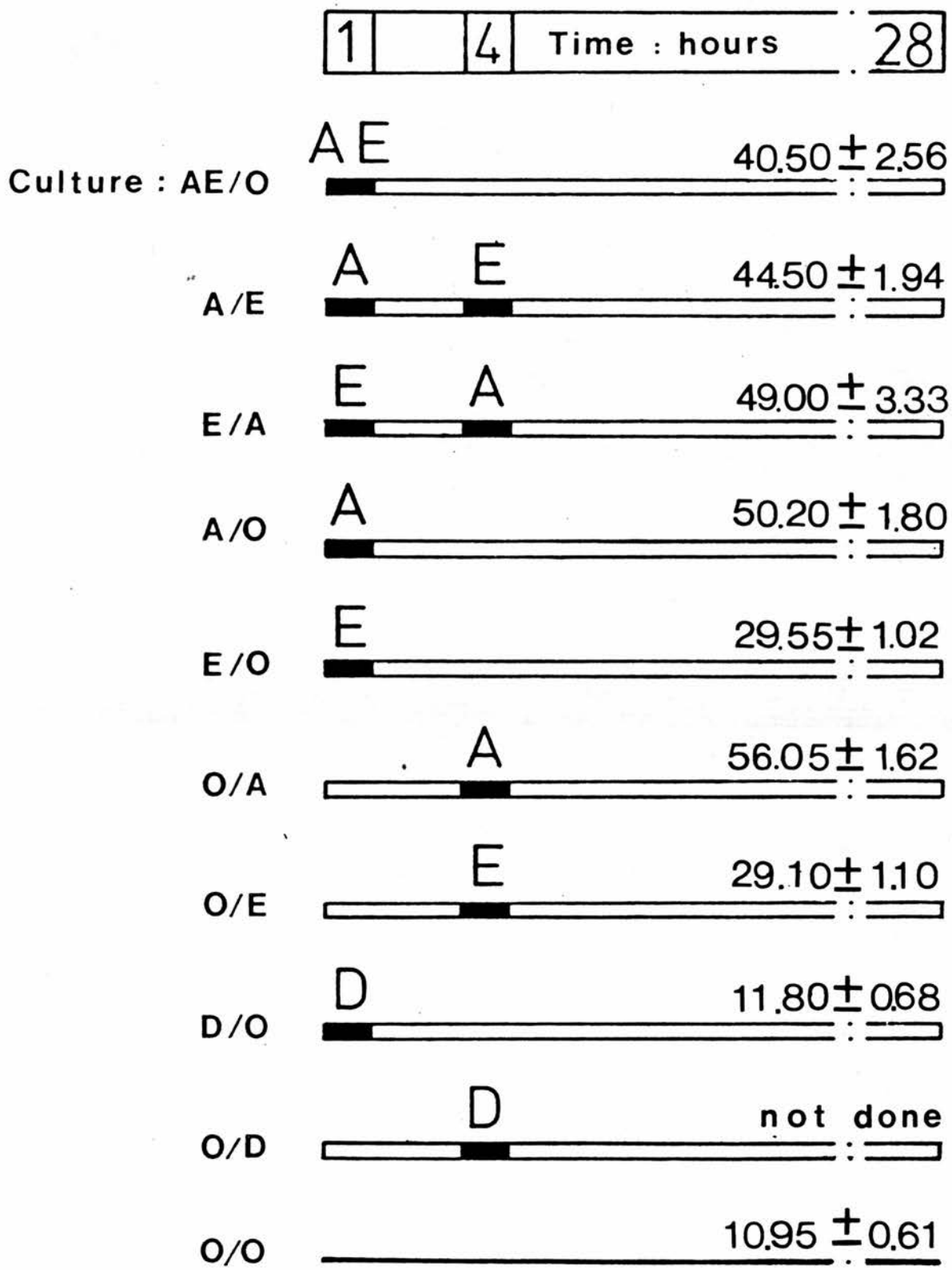


Table 4.5.2. ANT + ENU Induced SCEs ± Standard Error

ANT and ENU were given together during the 1st hour of culture or assplit doses separated by 2 hours in mutagen-free conditions. Cells were harvested at 28 hours.

ANT concentration: 25×10^{-7}M; ENU concentration: 5×10^{-4}M

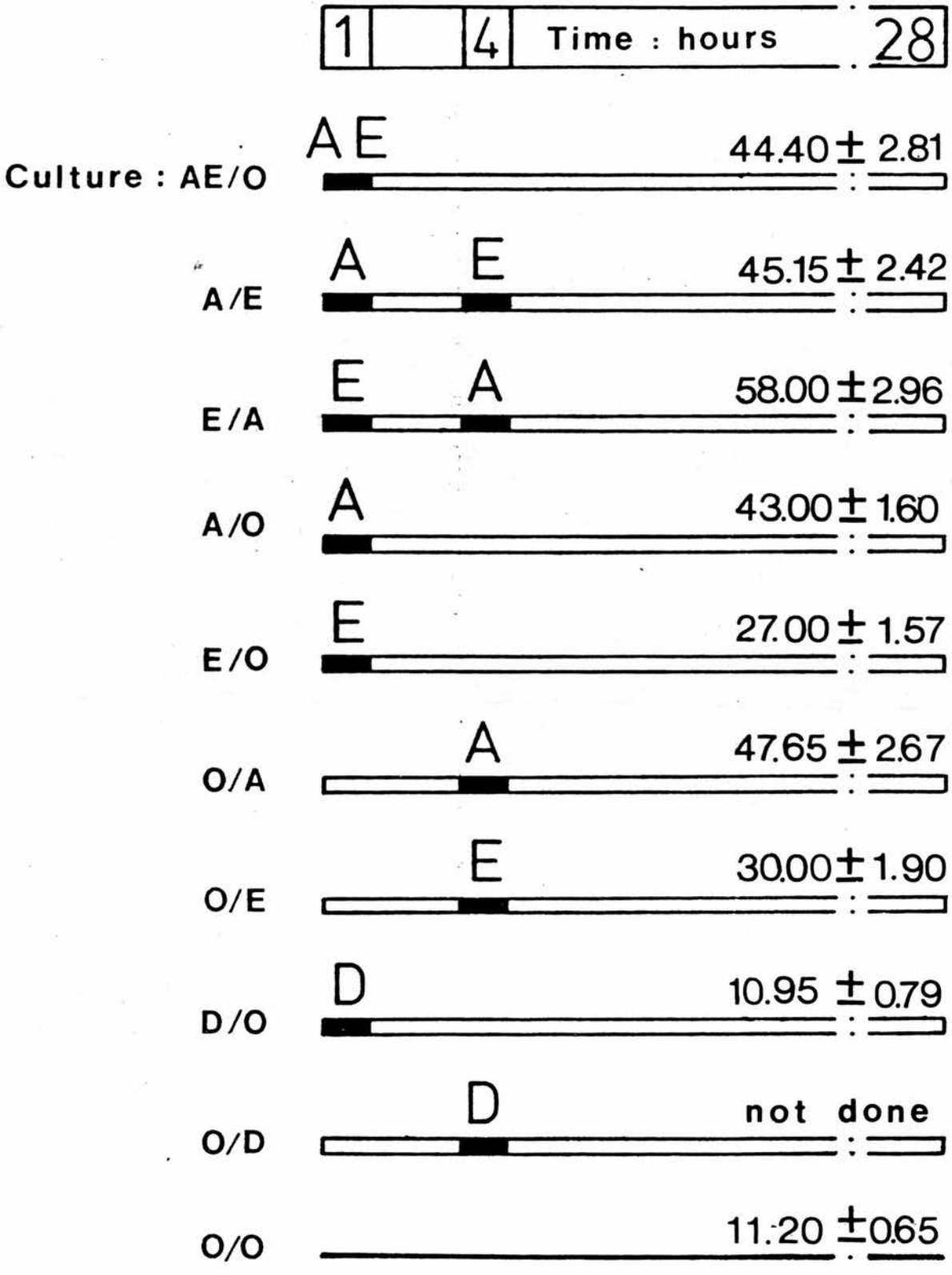


Table 4.5.3. ANT + ENU Induced SCEs ± Standard Error

ANT and ENU were given either together during the 1st hour of culture or as split doses separated by 2 hours in mutagen-free conditions. Cells were harvested at 28 hours.

ANT concentration: 12.5×10^{-7}M; ENU concentration 4×10^{-4}M

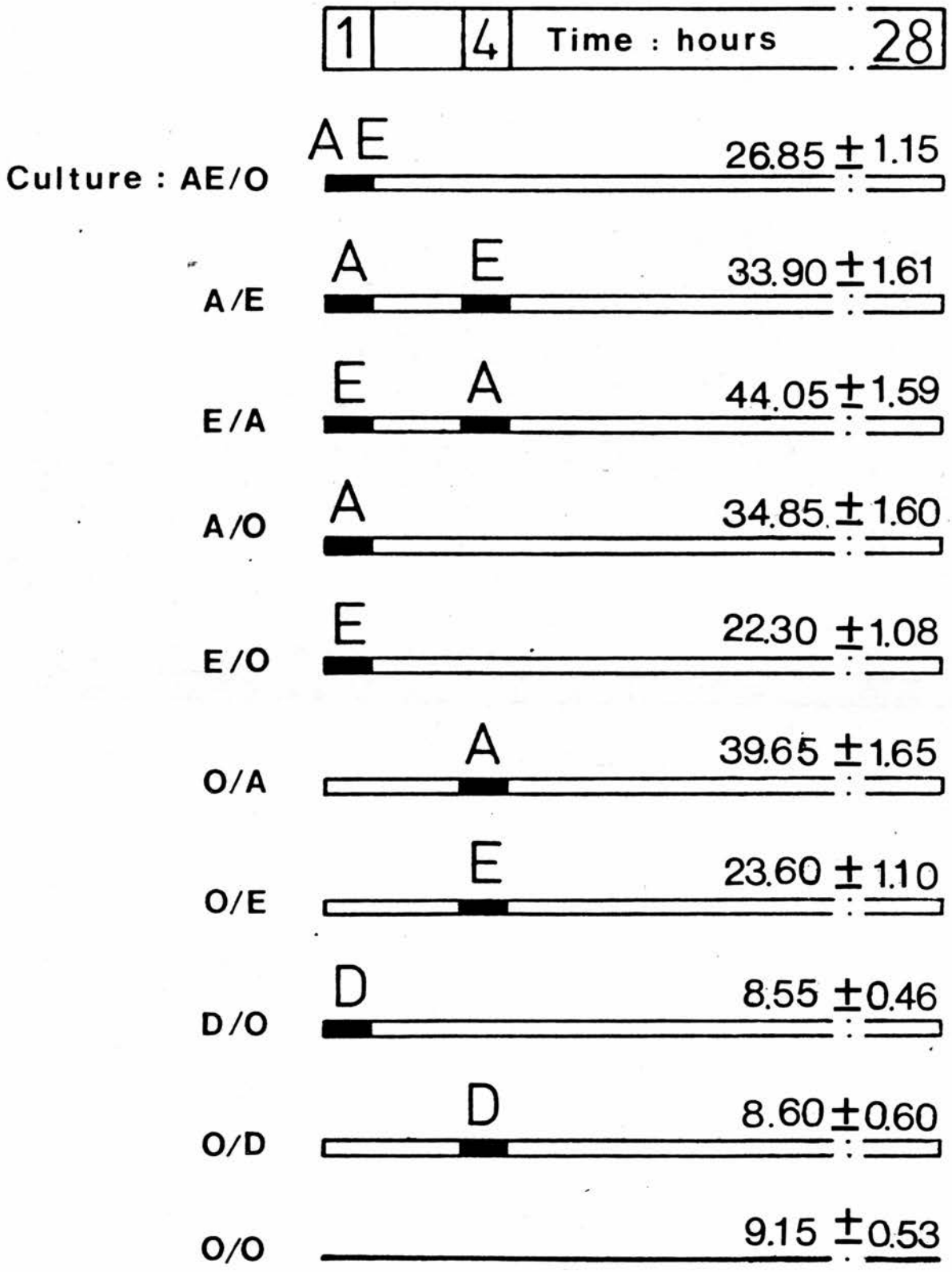


Table 4.5.4. ANT + ENU Induced SCEs ± Standard Error.

ANT and ENU were given either together during the 1st hour of culture or as split doses separated by 2 hours in mutagen-free conditions. Cells were harvested at 28 hours.

ANT concentration: 6×10^{-7}M; ENU concentration: 4×10^{-4}M

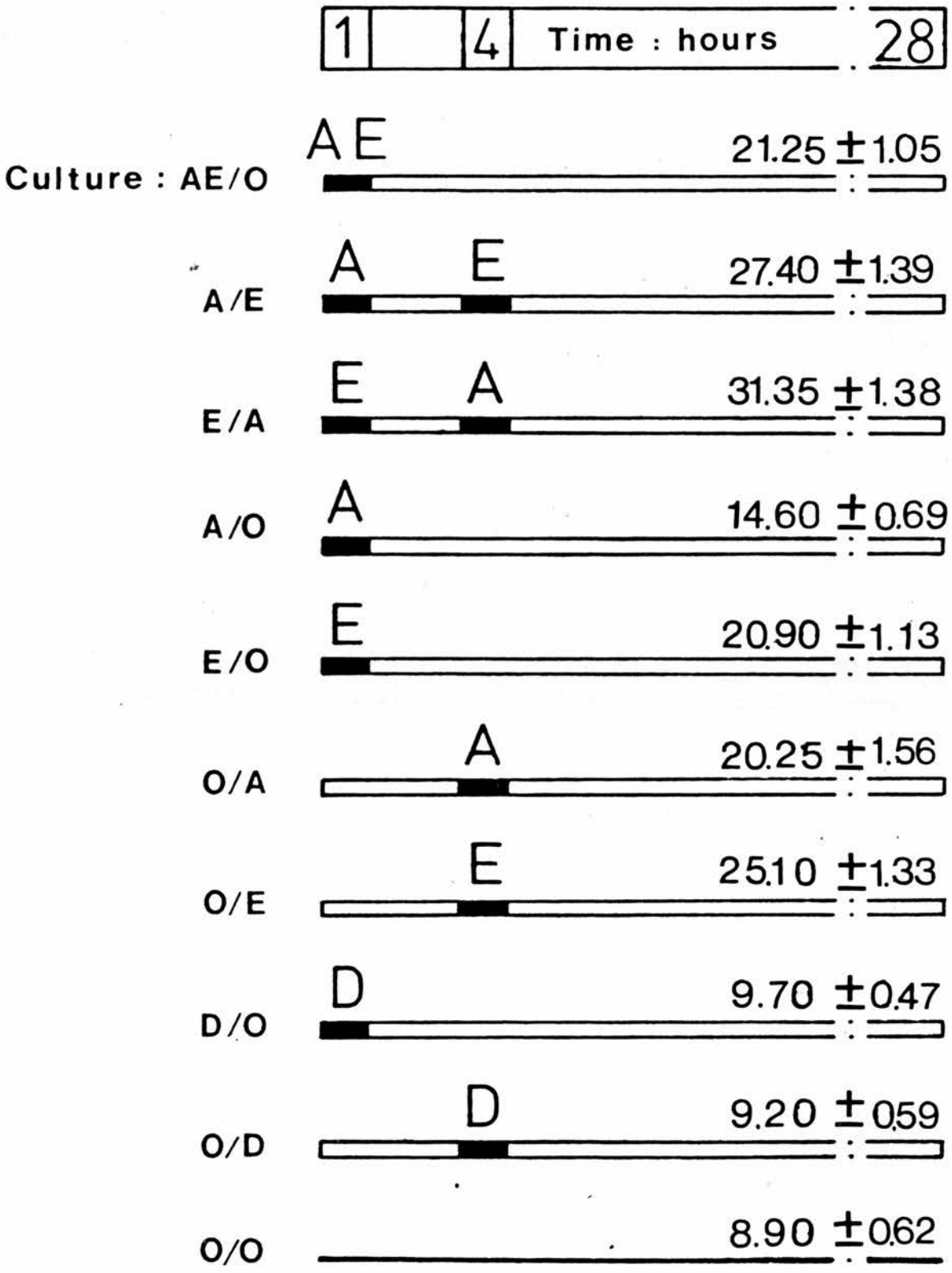


Table 4.5.5. Results of the Statistical Analysis (t test) of ANT + ENU Split Dose Experiments.

E	T	Observed SCE/cell (s^2)	Expected SCE/cell (s^2)	t	p
1	AE/0	40.05 (143.05)		12.64	p<0.001
	A/E	44.50 (75.11)	71.60 (87.83)	11.73	p<0.001
	E/A	49.00 (222.42)		8.47	p<0.001
2	AE/0	44.40 (157.62)		5.79	p<0.001
	A/E	45.15 (117.19)	62.75(161.71)	5.69	p<0.001
	E/A	58.00 (174.84)		1.48	p>0.050
3	AE/0	26.85 (26.34)		11.73	p<0.001
	A/E	33.90 (51.88)	51.43 (80.85)	8.08	p<0.001
	E/A	44.05 (50.47)		3.42	p<0.001
4	AE/0	21.25 (22.09)		5.28	p<0.001
	A/E	27.40 (38.57)	31.16 (70.61)	1.87	p>0.050
	E/A	31.35 (38.13)		0.09	p>0.050

Column E lists the experiment numbers (which refer to the Tables in this Section)
Column T gives the treatment protocol (see Tables in this Section for Experimental Protocol)
Column t gives the t value obtained from the t-test analysis
Column p gives the significance levels.

For a description of the way in which the expected SCE/cell values were derived, see p. 65.

Preliminary Discussion of ANT/ENU Results

From the results obtained in this Section, it is clear that the results obtained in Experiment 6, Section 4, were not spurious results and that ANT and ENU combined treatments have a blocking effect on SCE induction. One can fairly safely rule out the possibility that ANT and ENU had reacted extra-cellularly to give inert products because (i) ENU plus ANT doses of 16 and 8 x 10^{-9}M had an additive effect on SCE induction (Table 4.4.6.); (ii) ANT/ENU split dose experiments 1, 2, and 3 showed a significant reduction in the observed SCE frequencies relative to the expected SCE frequencies (Tables 4.5.1. to 4.5.3 and 4.5.5.).

The reasons for the discrepancy between the observed and the expected results are unclear. It is note-worthy that E/A split dose treatment always gave a higher SCE response than A/E split dose treatment. This may be partly accounted for by the fact that ENU reacts very quickly with DNA: a 1-hour treatment with ENU induces the same number of SCEs/cell as a 24-hour treatment with the same dose of ENU (Tables 4.3.1, 4.4.6. and 4.5.1-4.5.4). ANT, on the other hand, has much slower binding kinetics (Hurley et al., 1979a; also demonstrated by the fact that a 24-hour exposure to 64 x 10^{-9}M ANT induces approximately 50 SCEs/cell whereas a 1-hour exposure requires 25 x 10^{-7}M ANT to induce approximately the same number of SCEs/cell). However, these considerations do not contribute to explaining why combined treatment of ANT and ENU had an "inhibitory" effect on SCE induction. An explanation of this effect will be offered in the General Discussion of this Chapter.

General Discussion for Chapter 4

The results presented in this Chapter indicate that O-6-alkylG cannot be the only DNA lesion involved in the generation of SCE. The qualitative and quantitative differences in DNA lesions produced by the agents studied are so large that one would not expect an interactive effect on the production of SCE when mutagens are administered in pairs. One would expect combined treatments to be additive with respect to SCE induction if (i) there is no competition for "SCE sites" and (ii) lesions produced by one agent in the pair are repaired *via* a different pathway from lesions produced by the other agent in the pair. This expectation was met for all pairs of mutagens except for MMS-and-X-rays and ANT-and-ENU.

MMS plus X-ray interactions have been extensively discussed in pages 41-43 of this Chapter. It was concluded that the repair systems operating on MMS-induced lesions and X-ray-induced lesions might "cross react" and thus lead to fewer than expected SCEs when MMS and X-rays are given in combination.

The finding that ANT plus ENU combined treatment yields fewer than expected SCEs is rather more difficult to explain than the MMS/X-ray results because very little information is available in the literature on anthramycin. Buckley et al. (1979) reported that rats fed on a diet supplemented with the carcinogen acetyl-aminofuorene exhibited enhanced repair (in liver) of O-6-methylG resulting from an injection with dimethylnitrosoamine. Though their interpretations of their findings are subject to debate (Cleaver and Kaufmann, 1980), their results raise an interesting possibility. In 1976 Westra et al. isolated the persistent DNA-acetyl-aminofluorene adduct which had been reported by Kreik (1972) and showed that it was N-2-(2acetyl-aminofluoren-3-yl)guanine. Although this product accounts for only 20% of the DNA adducts identified after treatment with acetyl-aminofluorene, it persists for over 8 weeks in rat liver (Kreik, 1972). Removal of ANT-DNA adducts is also a slow process (Hurley and

Petrusek, 1979). It is tempting to draw an analogy between the findings of Buckley et al. (1979) and the one obtained in the present study and suggest that perhaps ANT induces an enzyme capable of rapidly removing the O-6-ethylG produced by ENU. The decrease below the expected SCE frequency would be significant for ANT plus ENU combined treatments but not for ANT plus EMS combined treatments because the amount of O-6-ethylG produced by EMS is 25 times lower than that produced by ENU (Sun and Singer, 1975).

The explanation advanced in the foregoing paragraph accounts for the observation that ENU/ANT split dose treatment yields higher SCE frequencies than ANT/ENU split dose treatment since cells exposed first to ENU would have time to synthesize some DNA (and presumably initiate SCE) before the O-6-ethylG excision enzyme dependent on ANT addition (which occurs at hour-4) could remove the ethylated bases. Why ANT + ENU/0 induced SCE frequencies are always lower than ANT/ENU induced SCE frequencies is not clear. At present no reasonable explanation involving enzyme induction can be offered. The observation that ANT + ENU combined treatment induces SCE frequencies within the expected range at ANT concentrations of 16 and 8 x 10^{-9}M cannot be explained. These results await more data on the repair of ANT-induced DNA lesions.

In conclusion, it appears from the findings obtained in this study that O-6-alkylG is certainly not the only lesion involved in the generation of SCE by alkylating agents. The formation of covalently bound ANT-DNA complexes at the N-2 position of guanine (Hurley and Petrusek, 1979) results in the generation of many SCEs per cell. ANT is an interesting drug in that, like 7,12-dimethylbenz[a]anthracene (Jeffrey et al., 1976a), acetyl-aminofluorene(Westra et al. 1976), benzo[a]pyrene (Osborne et al., 1976, Weinstein et al., 1976), it forms adducts with DNA at the N-2 position of guanine. ANT however does not require metabolic activation for SCE-induction (since it induces SCE in CHO cells which require S9-mix to activate pro-mutagens). In this respect ANT behaves like the direct-acting alkylating agent

7-bromomethylbenz[a]anthracene which also alkylates the N-2 position of guanine (Dipple et al., 1971, Rayman and Dipple, 1973a,b) as well as other sites. The results obtained for ANT + ENU combined treatment should be more precisely interpretable once biochemical data are obtained for ANT repair pathways.

CHAPTER 5

DOWN'S SYNDROME: EFFECTS OF BLEOMYCIN AND MITOMYCIN C ON SCE INDUCTION AND CELL CYCLE KINETICS

Introduction

Cells (lymphocytes and fibroblasts) from individuals with Down's syndrome (DS), characterized karyotypically by the presence of trisomy 21, exhibit a greater response to the induction of chromosomal aberrations by ionizing radiation than cells from karyotypically normal individuals (Dekaban et al., 1966, Evans and Adams, 1973, Kučerová, 1967, Kučerová and Poliková, 1978, Lambert et al., 1976, Sasaki et al., 1970, Sasaki and Tonomura, 1969). Surprisingly little work has been done on DS response to agents other than ionizing radiation. O'Brian et al. (1971) reported DS hypersensitivity to 7,12-dimethylbenz[a]anthracene-induced aberrations. DS lymphocytes were also shown to be hypersensitive to aberration-induction by N-methyl-N-nitrosourea (Kaina, 1977, Kaina et al., 1977) and UV (Lambert et al., 1976). However, Kučerová and Poliková (1978) reported normal levels of mitomycin C (MMC)-induced aberrations and SCEs in DS lymphocytes. In view of the conflicting findings on DS sensitivity to chromosomal aberration induction by chemical mutagens, the present study was undertaken to investigate the effects of bleomycin (BLM), an agent that has some similarity to X-rays in its effects on chromosomes, on SCE induction in DS lymphocytes (for a description of the cytogenetic effects of BLM see Chapter 3, pp. 26, 27). As was dicussed in Chapter 3, the association of SCEs and aberrations is by no means a strong one. However, since aberrations are sometimes associated with SCE, it seemed of

interest to investigate whether radiosensitivity of DS lymphocytes could be matched by an enhanced response after exposure to the radio-mimetic agent BLM.

DS lymphocyte sensitivity to MMC induced SCEs was included as a negative control (after the findings of Kučerová and Polikovᡠ, 1978). In order to ensure that the cell populations sampled for SCE scoring were comparable, the frequencies of first, second and further divisions (M1, M2, and $\geq$ M3, respectively) were scored in each culture. This part of the study seemed of particular importance because Taylor et al. (1979) reported that ataxia telangiectasia (AT) fibroblasts are more sensitive to the cytotoxic and clastogenic effects of BLM than normal cells. AT cells have also been shown to be hypersensitive to cell killing and aberration induction by ionizing radiation (Higuraishi and Conen, 1973, Hoar and Sargent, 1976, Paterson et al., 1975, Taylor et al., 1975, 1976).[1]

Materials and Methods

Drs. William Campbell and Marjorie Newton (MRC Clinical and Population Cytogenetics Unit, Edinburgh) kindly provided venous blood samples from five male DS patients and their carefully matched chromosomally normal but mentally retarded male controls from the same institution. One individual in this study was receiving medication at the time of blood sampling. This subject, DS 3, was a diabetic receiving insulin and metformin in standard therapeutic doses. All five DS subjects were of the standard type of trisomy 21. One of the controls, unfortunately, was ascertained to be a Kleinfelter individual in the course of this study (control 2). Sasaki et al. (1970) reported that lymphocytes of individuals with the XXY chromosome constitution showed an enhanced response to aberration induction

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1. The reader is referred to recent papers by Bridges and Harnden (1981) and Paterson and Smith (1979) for futher discussion of AT.

by ionizing radiation. However, Evans and Adams (1973) challenged this claim and reported that XXY and XY cells had similar responses to X-irradiation. In this study the XXY cells behaved in much the same way as XY cells in their response to BLM and MMC.

From each blood sample twelve whole blood cultures were set up (see Chapter 2). One culture from each subject remained untreated and was used as the control for that subject. Five cultures from each subject were exposed for 72 hours each to one of the following MMC concentrations: 3×10^{-8}M, 5×10^{-8}M, 8×10^{-8}M, 9×10^{-8}M and 1×10^{-7}M. The remaining six cultures from each subject were exposed for 72 hours each to one of the following BLM concentrations: 1.25×10^{-7}M, 2.5×10^{-7}M, 5×10^{-7}M, 1×10^{-6}M, 1.5×10^{-6}M and 2×10^{-6}M. Cells were harvested at 72 hours as described in Chapter 2. It is important to note that all 120 cultures were set up and harvested at the same time with a view to eliminating small but perhaps noticeable inter-experimental differences.

SCEs were scored for four DS subjects and their matched controls (all 12 cultures of one DS having failed to transform). The frequency of M1, M2 and $\geq$M3 (first, second and further divisions, respectively) cells were scored for three DS subjects and their matched controls.

Results and Statistical Analysis of the Data

The results for BLM and MMC SCE induction in DS and control lymphocytes are presented in Tables 5.1. and 5.2. DS lymphocyte cultures are referred to as DS-1, DS-2, DS-3 and DS-4. Lymphocyte cultures C-1, C-2, C-3 and C-4 refer to lymphocyte cultures established from blood samples drawn from the respective controls to the DS individuals. A t test analysis was performed to test for differences in response to BLM and MMC SCE-induction responses between DS and control lymphocytes. The results of this analysis are also tabulated in Tables 5.1. and 5.2.
Tables 5.3. and 5.4. list the frequencies of M1, M2, and $\geq$ M3 cells in each culture.

Table 5.1. Mean SCE/Cell ± Standard Error (S.E.) of Down's (DS) and Control (C) Lymphocytes Exposed to BLM (n=20 cells

BLM Treatment x 10^{-7} M	Down's Syndrome SCE/cell ± S.E.	Control SCE/cell ± S.E.	S
0	DS-1 5.95 ± 0.42	C-1 6.15 ± 0.48	
	DS-2 5.20 ± 0.55	C-2 7.45 ± 0.70	t= 2.89
	DS-3 5.10 ± 0.47	C-3 6.95 ± 0.60	p<0.010
	DS-4 4.65 ± 0.53	C-4 4.90 ± 0.49	
1.25	DS-1 7.00 ± 0.81	C-1 5.55 ± 0.52	
	DS-2 4.85 ± 0.48	C-2 5.45 ± 0.63	t= 0.47
	DS-3 6.65 ± 0.75	C-3 5.95 ± 0.43	p>0.050
	DS-4 5.15 ± 0.41	C-4 5.90 ± 0.60	
2.50	DS-1 6.80 ± 0.64	C-1 5.55 ± 0.54	
	DS-2 6.75 ± 0.61	C-2 5.30 ± 0.48	t= 2.34
	DS-3 6.20 ± 0.52	C-3 5.90 ± 0.41	p<0.020
	DS-4 6.45 ± 0.60	C-4 S.F.	
5.00	DS-1 5.85 ± 0.59	C-1 6.85 ± 0.58	
	DS-2 5.55 ± 0.57	C-2 5.65 ± 0.44	t= 0.64
	DS-3 7.15 ± 0.77	C-3 5.10 ± 0.52	p>0.050
	DS-4 S.F.	C-4 S.F.	
10.00	DS-1 7.65 ± 0.66	C-1 7.20 ± 0.69	
	DS-2 6.55 ± 0.57	C-2 6.80 ± 0.72	t= 0.26
	DS-3 5.75 ± 0.65	C-3 7.00 ± 0.83	p>0.050
	DS-4 7.50 ± 0.72	C-4 S.F.	
15.00	DS-1 6.80 ± 0.78	C-1 5.17[1] ± 0.55	
	DS-2 7.53[2] ± 0.94	C-2 6.80 ± 0.48	t=1.07
	DS-3 6.55 ± 0.80	C-3 7.10 ± 0.65	p>0.050
	DS-4 7.00 ± 0.62	C-4 6.50 ± 0.58	
20.00	DS-1 U	C-1 U	
	DS-2 U	C-2 6.70 ± 0.77	t= 3.70
	DS-3 7.85 ± 0.62	C-3 6.50 ± 0.73	p<0.001
	DS-4 8.75 ± 0.57	C-4 5.20 ± 0.49	

The S column lists the t values and the significance levels
S.F., staining failure; [1], n=18cells; [2], n=15cells;
U, unscorable M2 cells (very damaged).

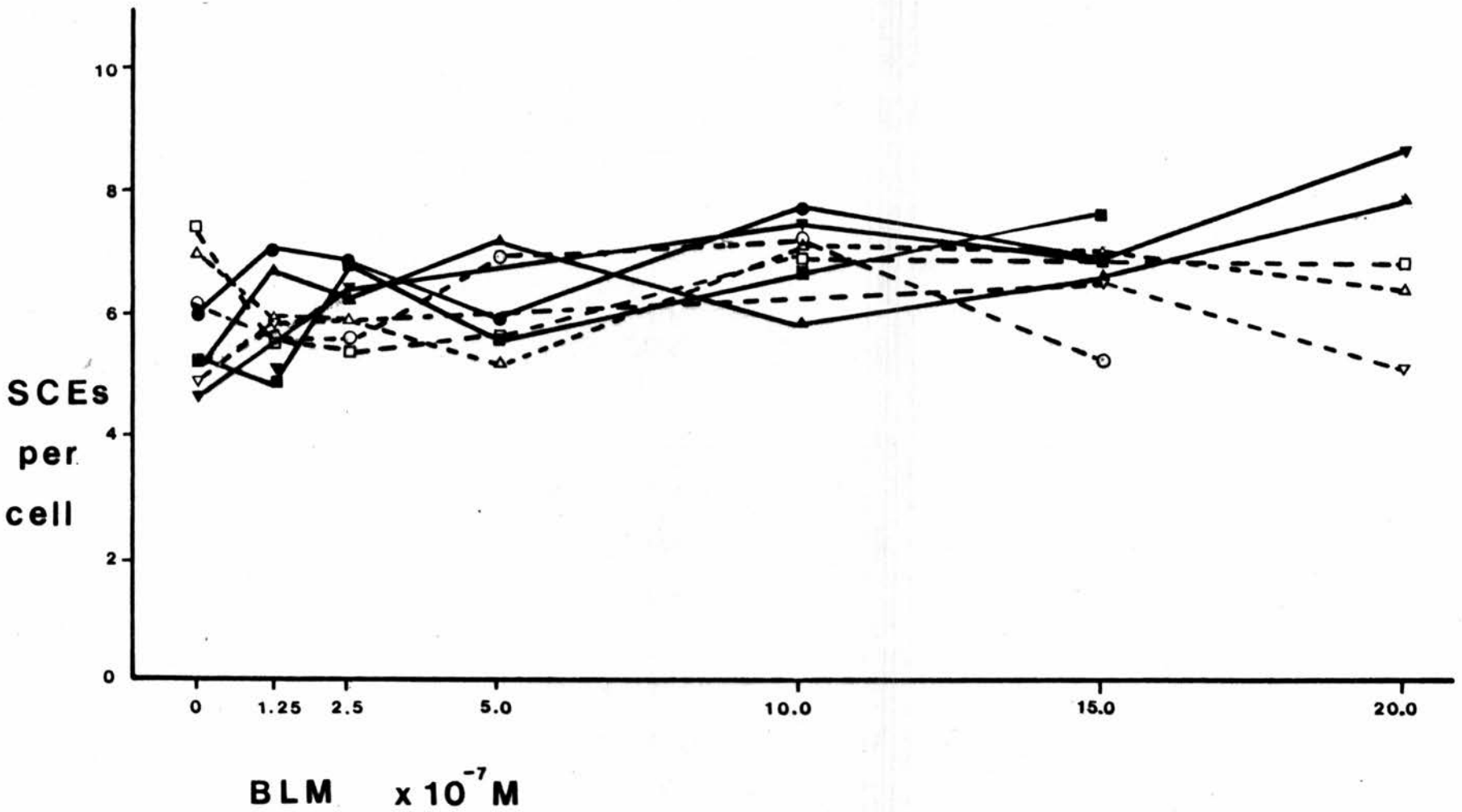

SCEs
per
cell
10
8
6
4
2
0
0
1.25
2.5
5.0
10.0
15.0
20.0
BLM x 10⁻⁷ M

Table 5.2. Mean SCE/Cell ± Standard Error (S.E.) of Down's (DS) and Control (C) Lymphocytes Exposed to MMC (n=20 cell

MMC Treatment x 10^{-8}M	Down's syndrome SCE/cell ± S.E.		Control SCE/cell ± S.E.		S
0	DS-1	5.95 ± 0.42	C-1	6.15 ± 0.48	
	DS-2	5.20 ± 0.55	C-2	7.45 ± 0.70	t= 2.89
	DS-3	5.10 ± 0.47	C-3	6.95 ± 0.60	p<0.010
	DS-4	4.65 ± 0.53	C-4	4.90 ± 0.49	
3.00	DS-1	21.35 ± 0.97	C-1	17.90 ± 1.07	
	DS-2	21.45 ± 1.08	C-2	17.85 ± 0.89	t= 4.11
	DS-3	20.80 ± 1.04	C-3	19.30 ± 1.19	p<0.001
	DS-4	19.35 ± 0.93	C-4	16.15 ± 0.82	
5.00	DS-1	29.05 ± 1.41	C-1	24.50 ± 1.39	
	DS-2	27.25 ± 1.29	C-2	26.80 ± 1.01	t= 0.58
	DS-3	25.85 ± 1.13	C-3	29.20 ± 1.12	p>0.050
	DS-4	23.50 ± 1.07	C-4	23.10 ± 0.91	
8.00	DS-1	43.25 ± 1.87	C-1	36.35 ± 2.05	
	DS-2	34.70 ± 1.33	C-2	33.10 ± 0.91	t= 0.64
	DS-3	28.50½ ± 1.33	C-3	35.10 ± 1.92	p>0.050
	DS-4	29.35 ± 1.22	C-4	27.95 ± 1.10	
9.00	DS-1	46.75 ± 1.74	C-1	38.85 ± 1.51	
	DS-2	38.65 ± 1.29	C-2	36.35 ± 1.63	t= 3.60
	DS-3	45.05 ± 1.62	C-3	42.95 ± 2.00	p<0.001
	DS-4	38.85 ± 1.19	C-4	33.80 ± 1.11	
10.00	DS-1	U	C-1	44.00 ± 1.84	
	DS-2	45.05 ± 2.02	C-2	37.45 ± 1.32	t= 1.94
	DS-3	46.35 ± 1.61	C-3	47.15 ± 2.62	p>0.050
	DS-4	39.00 ± 1.17	C-4	32.90 ± 1.81	

The S column lists the t values and the significance levels
½, n = 10 cells, U, unscorable M2 cells (very damaged).

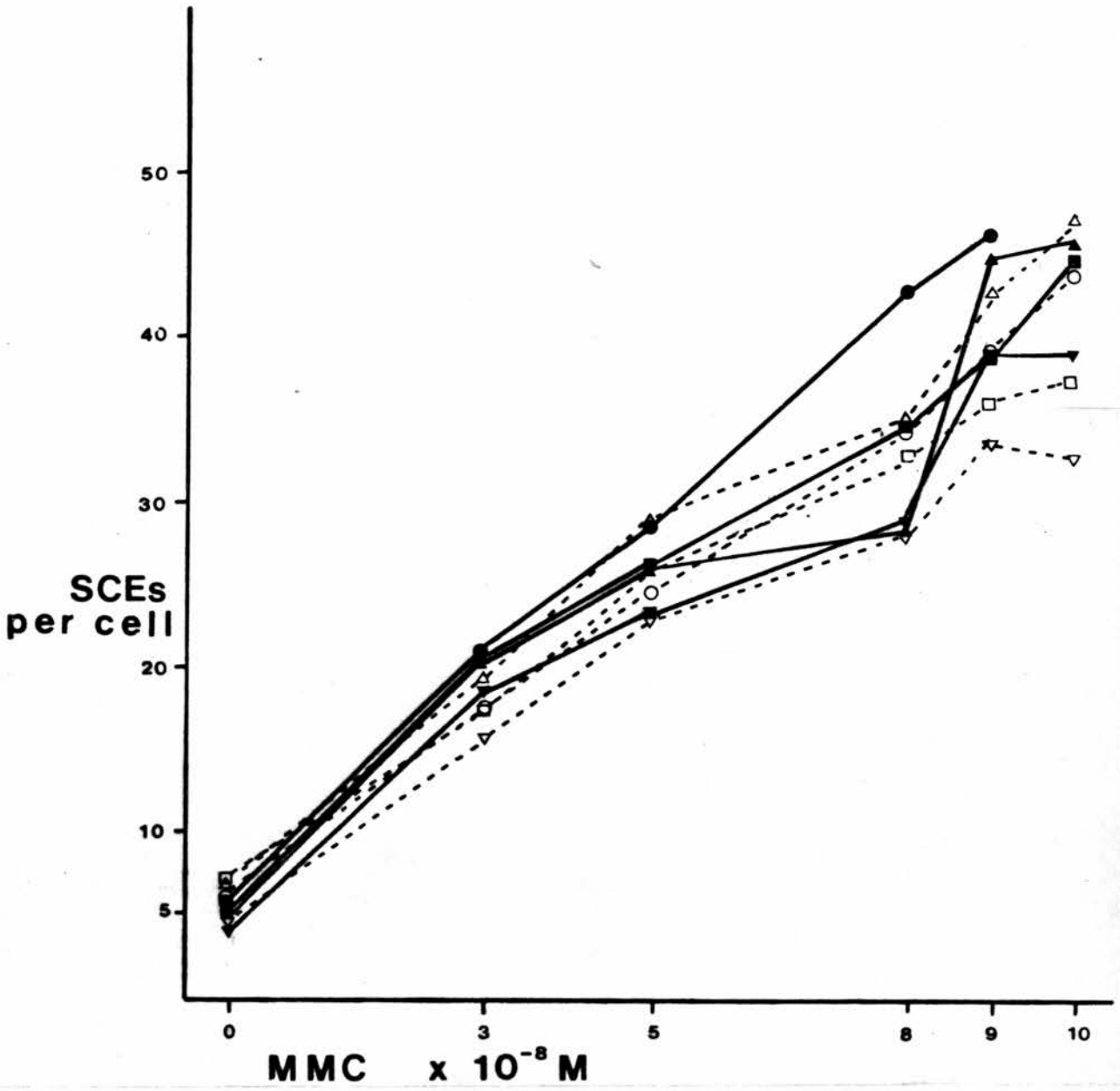


Figure 5.2. Mean SCE/Cell in Cultures of Down's Syndrome and Control Lymphocytes Exposed to MMC

Solid symbols: Down's syndrome cultures

- ● DS-1
- ■ DS-2
- ▲ DS-3
- ▼ DS-4

Open symbols: Control cultures

- ○ C-1
- □ C-2
- △ C-3
- ▽ C-4

Table 5.3. Percent Frequencies of First, Second, and Further Divisions (M1, M2, ≧ M3, respectively) in BLM-Treated Cultures of Three Down's (DS) Subjects and Their Matched Controls (C). The data are based on observation of 50 randomly selected metaphases.

BLM Treatment x 10^{-7}M	% Frequency Down's Syndrome				% Frequency Control			
		M1	M2	≧M3		M1	M2	≧M3
0	DS-1	28	42	30	C-1	10	32	58
	DS-2	16	34	50	C-2	8	26	66
	DS-3	14	34	52	C-3	34	28	38
1.25	DS-1	20	40	40	C-1	6	34	60
	DS-2	16	54	30	C-2	10	36	54
	DS-3	22	42	36	C-3	20	40	40
2.50	DS-1	14	44	42	C-1	22	44	34
	DS-2	8	18	74	C-2	22	48	30
	DS-3	6	54	40	C-3	52	26	22
5.00	DS-1	34	38	28	C-1	26	34	40
	DS-2	26	36	38	C-2	18	40	42
	DS-3	16	44	40	C-3	22	50	28
10.00	DS-1	6	38	56	C-1	10	32	58
	DS-2	20	28	52	C-2	14	26	60
	DS-3	12	22	66	C-3	10	36	54
15.00	DS-1	16	42	42	C-1	22	26	52
	DS-2	24	34	42	C-2	4	14	82
	DS-3	18	24	58	C-3	16	36	48
20.00	DS-1	38	42	20	C-1	24	42	34
	DS-2	30	40	30	C-2	18	26	56
	DS-3	18	46	36	C-3	18	44	38

Table 5.4. Percent Frequencies of First, Second, and Further Divisions (M1, M2, ≧ M3, respectively) in MMC-Treated Cultures of Three Down's (DS) Subjects and Their Matched Controls (C). The data are based on observation of 50 randomly selected metaphases.

MMC Treatment x 10^{-8}M	% Frequencies Down's Syndrome				% Frequencies Controls			
		M1	M2	≧M3		M1	M2	≧M3
0	DS-1	28	42	30	C-1	10	32	58
	DS-2	16	34	50	C-2	8	26	66
	DS-3	14	34	52	C-3	34	28	38
3.00	DS-1	36	42	22	C-1	14	58	28
	DS-2	16	38	46	C-2	8	46	46
	DS-3	30	38	32	C-3	22	52	26
5.00	DS-1	34	36	30	C-1	30	58	12
	DS-2	34	38	28	C-2	20	52	28
	DS-3	24	48	28	C-3	32	42	26
8.00	DS-1	34	44	22	C-1	32	58	10
	DS-2	34	40	26	C-2	16	58	26
	DS-3	42	38	20	C-3	38	40	22
9.00	DS-1	38	40	22	C-1	36	54	10
	DS-2	46	38	16	C-2	28	56	16
	DS-3	36	44	20	C-3	40	44	16
10.00	DS-1	92	8	0	C-1	34	58	8
	DS-2	74	20	6	C-2	28	62	10
	DS-3	54	38	8	C-3	38	50	12

Discussion

In this study Down's syndrome lymphocytes exhibited significantly fewer SCEs per cell than control lymphocytes (Table 5.1). This observation is in disagreement with those of other authors (Kučerová and Poliková, 1978, Lezana et al., 1977, Yu and Borganokar, 1977). However, this difference may be a spurious one since the number of individuals (eight) sampled in this study was small.

After the completion of this study Crossen and Morgan (1980) published results indicating that DS-lymphocyte SCE frequency was significantly increased by 50 rad X-rays whereas 100 rad were required to produce a detectable increase in control lymphocytes. However, these authors irradiated the lymphocyte cultures at 44-hours after PHA stimulation. There is now good evidence from studies undertaken to compare cell cycle time in normal and DS lymphocytes and fibroblasts that DS cells cycle differently from normal cells (Kishi, 1977, Kukharenko et al., 1974, Paton et al., 1974, Segal and McCoy, 1973). As was pointed out in Chapter 1, the cell cycle stage in which cells are damaged is a critical factor in determining the SCE frequency observed. Therefore, it is possible that the hypersensitivity of DS lymphocytes to X-ray induced SCEs reported by Crossen and Morgan might be accounted for by differences in cycling states of DS and normal lymphocytes at the time of irradiation.

As expected from the findings of Kučerová and Poliková (1978), DS lymphocytes exhibited no consistent increase in MMC-induced SCEs (Table 5.2., Figure 5.2.). The results in the present study also indicate that there is no differential effect of MMC on the cell cycling kinetics between DS lymphocytes and similarly treated control lymphocytes (Table 5.4.).

With regard to BLM-induced SCEs, the response of DS lymphocytes did not consistently differ from that of control lymphocytes (Table 5.1., Figure 5.1.). The results obtained for the effects of BLM on cell cycle kinetics show that large

inter- and intra-individual differences exist in cultures sampled at a given time (Table 5.3.). Results obtained by Purrott et al. (1980) suggest that X-rays cause drastic shifts in M1, M2, and M3 populations of lymphocytes. This effect has also been demonstrated by Craig-Holmes and Shaw (1977) for lymphocytes exposed to chemical mutagens. These authors also reported large inter-individual differences with respect to MMC-induced cell cycle delay. Furthermore, inter- and intra-individual differences have been reported in FPG cell cycle studies in untreated lymphocytes (Bianchi and Lezana, 1976, Crossen and Morgan, 1977, Craig-Holmes and Shaw, 1977). The results obtained in this study are in agreement with the above-mentioned findings. Although it was not possible statistically to analyse the data because of the small sample size (three DS and three control individuals), it is clear that the proportion of second division cells available for SCE analysis varies from donor to donor. Futhermore, the proportion of second division cells in cultures from one donor does not consistently decrease with increasing concentration of mutagen and in fact sometimes exceeds the proportion of cells in the untreated culture (Tables 5.3. and 5.4.). This observation raises the following questions: are inter-culture differences with respect to the proportion of second division cells due to

(1) "normal" variation?
(2) differential cell killing of a subpopulation of lymphocytes?
(3) alterations in the cell cycle length (which may [Ockey, 1977] or may not [Beek and Obe, 1979] affect SCE frequencies)?

It would be of interest to investigate these questions in order to ensure that valid comparisons can be made between groups of individuals.

In conclusion, lymphocytes of DS individuals do not differ significantly from lymphocytes of control individuals in their response to SCE induction by BLM and MMC. In view of the pronounced inter- and intra-individual differences

with respect to the proportion of second division cells available for SCE scoring, further studies ought to be carried out in order to ensure that SCE frequencies of DS and control lymphocytes are directly comparable. However, due to the fact that DS and control donors could not be regarded strictly as willing participants in the study, it was considered unethical to continue these experiments.

CHAPTER 6

GAMMA-IRRADIATION OF HUMAN PERIPHERAL LYMPHOCYTES: EFFECTS OF LOW AND PROLONGED IRRADIATION ON SCE INDUCTION

Introduction

X-rays have been shown to induce chromosomal aberrations much more efficiently than SCEs (Perry and Evans, 1975, Pant et al., 1976). Detection of low levels of ionizing radiation induced damage has been successfully investigated *in vitro* using aberrations as an index of damage (Kučerová et al., 1972, Luchnik and Sevankaev, 1976, Pant et al., 1976, Schmickel, 1967). In a paper presented at the 14th International Congress of Genetics, Moscow, 1978, Serra et al. reported a significant average increase of SCEs per cell in lymphocytes of a group of subjects exposed continuously to small doses of radiation. The report by Serra et al. was surprising in the context of previous demonstrations that acute X-ray exposure *in vitro* is inefficient in elevating SCE frequencies (Perry and Evans, 1975, Pant et al., 1976). Their finding, however, if confirmed could have far-reaching consequences both in terms of our understanding of mechanisms involved in SCE formation and, in particular, in terms of the use of the SCE end-point as a simple method for monitoring chronic exposure of man to low doses of X-rays.

An experiment was designed to investigate *in vitro* the *in vivo* observations of Serra et al. (1978). It also seemed worthwhile to investigate at the same time whether low but prolonged doses of radiation might increase the SCE frequency of cycling cells. The interest in this question was sparked by the finding of Perry and Evans(1975)

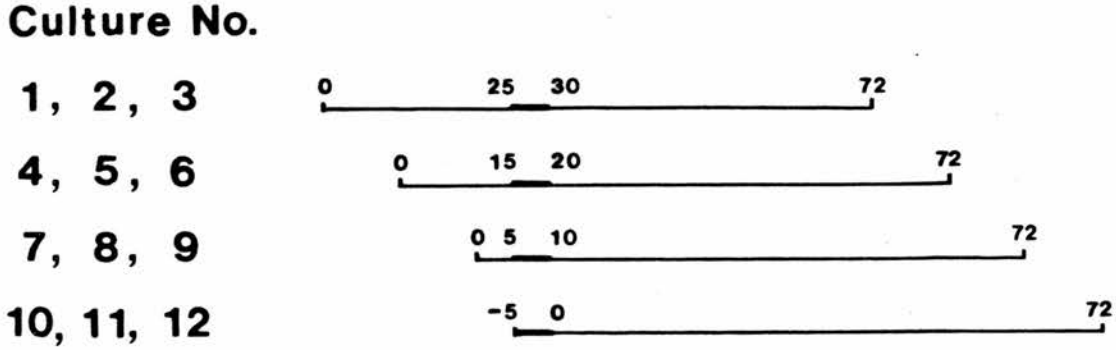
that in synchronized CHO cells, S-irradiated cells had a significantly higher SCE frequency of SCEs per cell than G1-irradiated cells.

Stimulated and unstimulated lymphocytes were continuously irradiated over a 5-hour period with a low gamma-emitting Cobalt 60 source. Although it is generally accepted that valid comparisons between in vivo and in vitro results can be achieved only if lymphocytes are irradiated at 37°C prior to PHA stimulation (Lloyd et al., 1975), the experiment described below was also designed to consider the SCE inducing effects of continuous low doses of radiation on stimulated lymphocytes in various stages (G1 to early-S) and therefore PHA was added to some of the cultures.

Materials and Methods

Venous blood was obtained from a healthy female donor. In order to irradiate all cultures at the same time it was necessary to set up and harvest the cells at 10-hourly intervals (see Chapter 2 for procedures). The irradiation and culture schedule are diagrammed below.

Figure 6.1. Irradiation and Culture Schedule.
Staggered setting up and harvest times were used in this experiment. Cultures were irradiated simultaneously for for five hours with a Cobalt 60 gamma source. PHA was added at time 0, colchicine at 70 hours and the cells were harvested at 72 hours.



The cultures were irradiated with a Cobalt 60 source (6.1 mrad/hour at 1m). All cultures were irradiated at the same time for five hours. In order to ensure continuation of "normal" processes during this long irradiation period, it was necessary to maintain the cells in warm medium. This was achieved by placing the culture vessels and the source in a 37°C water-bath. The source was encased in a specially-made perspex holder and positioned in the middle of the water-bath. The cultures were placed around the source at premarked locations. Dr. David Bonnet, of the MRC Cyclotron Unit, Edinburgh, very kindly carried out the irradiation, having calculated and checked the dose rate received by each culture. The table below gives details of the dose rate and range received by each culture.

Table 6.1. Dose Rate and Range of Gamma-Rays Received by Each Culture at a Given Distance from the Cobalt 60 Source (in a 37°C water-bath).

Culture No.	Distance From Source	Dose Rate	Dose Range
1, 4, 7, 10	17.20 cm	1 rad/hr	1.2 to 0.8 rad/hr
2, 5, 8, 11	8.80 cm	5 rad/hr	7.0 to 3.6 rad/hr
3, 6, 9, 12	4.45 cm	20 rad/hr	38 to 12 rad/hr

Owing to the now well-established properties of BUdR as a radiosensitizing agent (see Chapter 1), it was thought better to add BUdR immediately after irradiation. Exception was made for culture 1, 2, and 3 where BUdR was added at the time of PHA stimulation (hour-0) because it was expected that a large proportion of cells would be beginning DNA synthesis by 25 to 30 hours (Wolff, 1969). Care was taken to ensure that irradiation proceed in the dark in order to avoid photolysis of the BUdR-substituted DNA in cells which might have incorporated some BUdR (Ikushima and Wolff, 1974).

Table 6.2. Mean SCE/Cell ± Standard Error (S.E.) After a Five-Hour Exposure to Low Doses of Gamma-Rays.

Culture No	Irradiation time (hrs)	Total Dose	SCE/cell ± S.E. (n = 20 cells)
1	25 to 30	5 rad	6.40 ± 0.65
2	after PHA	25 rad	6.50 ± 0.39
3		100 rad	7.20 ± 0.56
4	15 to 20	5 rad	6.15 ± 0.48
5	after PHA	25 rad	7.10 ± 0.67
6		100 rad	7.00 ± 0.55
7	5 to 10	5 rad	6.55 ± 0.55
8	after PHA	25 rad	6.90 ± 0.55
9		100 rad	7.00 ± 0.61
10	5	5 rad	6.40 ± 0.54
11	before PHA	25 rad	5.20 ± 0.43
12		100 rad	5.40 ± 0.56
13	Control		6.00 ± 0.60

No significant difference between any of the mean SCE frequencies of irradiated cultures and the mean frequency of the control culture was found after a t-test analysis was performed on the data ($p>0.050$).

Discussion

Rapid repair of low LET radiation damage is a generally accepted phenomenon. Although different authors (Countryman and Heddle, 1976, Lett et al., 1967, Linieki et al., 1977, Prempree and Merz, 1969, Regan and Setlow, 1974, Wolff, 1972) disagree in their estimates of rejoining time (these differences are partly accounted for by variation in the irradiation protocol, the method used to quantitate rejoining time, and the type of cells used), rejoining time has not been reported to last longer than five hours (for G0-irradiated lymphocytes). Ionizing radiation damage is repaired by short-patch repair involving insertion of very few nucleotides (Regan and Setlow, 1974). Futhermore, it has been clearly demonstrated in vitro that the SCE frequency is not appreciably increased until high doses of radiation are used and that at these doses the increase in aberration frequency is much more pronounced (Abramovsky et al., 1978, Pant et al., 1976, Perry and Evans, 1975, Solomon and Bobrow, 1975). It should be pointed out that in all of these experiments irradiation was carried out on unifilarly BUdR-substituted chromatids and that it is possible that the observed SCEs were a result of the radiosensitizing effect of BUdR (e.g. Wolff and Fijtman, 1981, for further discussion see Chapter 1, Section C (i): BUdR-induced SCEs).

The results obtained in the present study demonstrate that there is no change in the SCE frequency of human lymphocytes irradiated in vitro with long exposures to low levels of gamma-radiation. This result is in disagreement with the in vivo results of Serra et al. (1978). However, in view of the enormous amount of evidence for rapid rejoining of radiation induced breaks, it seems unlikely that lesions induced in vivo (Serra et al., 1978) would remain unrepaired and "express" their existence through an increase in the SCE frequency after PHA stimulation in vitro. This contention is substantiated by the findings of Littlefield et al (1979) which appeared shortly after the completion of the present study. Littlefield et al. reported no increase

in the SCE frequency of GO-irradiated human lymphocytes. These authors pointed out that in vivo irradiation causes damage to GO lymphocytes which have not incorporated BUdR into their DNA. This is an important point in view of the fact that no increase in the SCE frequency was observed in G1 and early-first-S-irradiated lymphocytes (Table 6.2.).

Perry and Evans (1975) demonstrated a significant increase in SCE frequency of synchronized CHO cells irradiated during S over the SCE frequency of similarly irradiated G1 cells. The differences between the S-irradiation-SCE frequency and the G1-irradiation-SCE frequency were small but significant within the 0 to 100 rad dose range (Perry and Evans found no increase in the SCE frequency of G2-irradiated cells). Perry and Evans speculated that the lesions induced by radiation which resulted in SCEs were long-lived lesions, presumably not single strand breaks. However, these authors irradiated G1 cells which had already replicated once in the presence of BUdR and S cells which were incorporating BUdR for the second time. BUdR-substituted DNA is more sensitive than native DNA to radiation damage (e.g. Wolff and Fijtman, 1981). Furthermore, Kato (1980) presented convincing evidence for a direct correlation between the number of replication forks and SCE induction by fluorescent light which causes breaks in BUdR-substituted DNA strands. The enhanced SCE frequency in S-irradiated cells with respect to G1-irradiated cells can be more easily explained by a combination of BUdR radiosensitization and a "replication fork effect" than by the postulation of long-lived X-ray-induced lesions. The present findings support this conjecture. The failure to observe an increase in the SCE frequency of early-S-irradiated cells (25 to 30 hours after PHA stimulation) can be attributed to the fact that these cells had not previously incorporated BUdR and that the gamma-radiation doses were small and of low intensity. The G1-irradiated lymphocytes exhibited no increase in SCE frequency (Table 6.2.) presumably because they were not hypersensitive to radiation (since BUdR was added after irradiation of these cells) and because rapid-repair

could have intervened to rejoin any breaks present.

In conclusion, it is improbable that the SCE end-point could be used to monitor chronic exposure of man to low levels of ionizing radiation. It also seems likely that some of the X-ray-induced SCEs reported by other authors (Abramovsky et al., 1978, Pant et al., 1976, Perry and Evans, 1975, Solomon and Bobrow, 1975) could be accounted for by BUdR sensitization of DNA to X-ray-induced strand breaks.

CHAPTER 7

SCE INDUCTION BY HYDRALAZINE

Introduction

Hydralazine (HYD) is a widely used hypotensive agent which has the unfortunate side-effect of inducing in some patients symptoms which closely resemble those of systemic lupus erythematosus, SLE (Alarcón-Sergovia et al., 1965, Dustan et al, 1954, Condemi et al, 1967, Erikson et al., 1956, Muller et al., 1955). Acetylated derivatives of HYD are excreted in the urine (Reindenberg et al., 1973) and it has been suggested that HYD induced SLE might occur with higher frequency in slow-acetylators (Perry et al., 1970, Drayer and Reindenberg, 1977). However, it has also been suggested that the acetylator-type might be irrelevant to the processes which govern the induction of lupus by HYD (Zacest and Kock-Weser, 1972).

Shaw et al. (1979) reported that hydralazine causes base-pair substitution mutations in Salmonella. It seemed therefore of interest to investigate first whether HYD was capable of increasing SCE levels and second whether it would be possible to detect patients at risk of developing HYD induced SLE, perhaps through looking for an above-control sensitivity to SCE induction by HYD.

The data which were collected to answer the first question are presented and discussed in the reprint of a paper which appeared in Mutation Research (see Appendix). Having established that HYD induces small but consistent increases in SCE frequency, it seemed worthwhile to investigate the second question. An ethically acceptable

approach to answering this question presented itself when I was given blood samples from a patient with HYD induced lupus. The *in vitro* response of the lymphocytes of this patient to HYD was investigated.

Materials and Methods

Blood samples from the HYD induced lupus patient were kindly provided by Dr. Paul Williams (Western General Hospital, Edinburgh). At the time when these experiments were performed it was not possible to obtain blood from a sex and age matched control who had undergone HYD therapy without ill-effects but whose HYD course had been discontinued two years ago, as in the case of the patient described here. One of the controls, BF, was a healthy male (sex matched). The other control was donor 1, female, from the published experiments. Two independent experiments were performed. In both instances the protocol followed that detailed in the publication (see Appendix): the cells were exposed to HYD for the first 24 hours of culture and were harvested at 72 hours.

Results and Statistical Analysis

The results from the two experiments described above are presented in Table 7.1. A t-test was applied to the data in order to compare the response of the lymphocytes of the HYD patient with those of the controls' lymphocytes to SCE induction by HYD. The data from the two experiments were pooled for this analysis and are presented in Table 7.2.

The frequencies of first, second and further division cells (M1, M2, and $\geq$ M3, respectively) were scored for each culture to determine the effects of HYD on cell cycle kinetics. The results are presented in Table 7.3.

Table 7.1. In Vitro Induction of SCE in Lymphocytes of a Hydralazine Induced SLE Patient (SLE), His Sex Matched Control (BF), And Donor 1 from the Published Experiments.

HYD Treatment (M)	Mean SCE/Cell ± Standard Error (S.E.). Results from 2 independent experiments (n=20)					
	SLE SCE/Cell ± S.E.		BF SCE/Cell ± S.E.		Donor 1 SCE/Cell ± S.E.	
Controls	7.45 ± 0.75	8.00 ± 0.77	7.40 ± 0.84	9.40 ± 0.87	7.50 ± 0.63	7.50 ± 0.54
1×10^{-6}	7.95 ± 0.70	8.90 ± 1.13	11.25 ± 0.96	8.45 ± 0.71	9.20 ± 0.79	7.60 ± 0.65
5×10^{-6}	8.05 ± 0.53	9.10 ± 0.73	8.45 ± 0.81	8.00 ± 0.76	8.95 ± 0.87	7.70 ± 0.84
1×10^{-5}	10.70 ± 0.76	10.00 ± 1.04	7.75 ± 0.72	8.75 ± 1.01	8.35 ± 0.57	8.00 ± 0.74
5×10^{-5}	10.25 ± 0.81	11.45 ± 0.88	6.75 ± 0.75	9.95 ± 0.97	9.80 ± 0.62	7.85 ± 0.67
1×10^{-4}	11.55 ± 0.92	10.55 ± 0.62	10.20 ± 1.02	9.65 ± 0.75	10.25 ± 0.81	9.85 ± 0.59
5×10^{-4}	15.20 ± 1.35	12.10 ± 0.73	12.20 ± 0.65	11.00 ± 0.71	14.60 ± 0.75	11.80 ± 0.99
1×10^{-3}	19.40[1] ± 1.86	15.45 ± 1.56	17.00[2] ± 1.68	17.00 ± 0.91	14.90 ± 0.75	14.35 ± 1.17

[1], mitotic inhibition, only 5 cells scored; [2], mitotic inhibition, only 16 cells scored.

Table 7.2. In Vitro Induction of SCEs by HYD in Lymphocytes of a HYD Induced SLE Patient and Control (C) Lymphocytes – Data Pooled from Table 7.1.

HYD Treatment	Pooled SCE/cell (s^2)	Difference of means between HYD(-)/HYD(+)	SLE/C
0	SLE 7.725 (11.28) C 7.950 (11.59)		$p>0.050$
1×10^{-6}M	SLE 8.425 (17.38) C 9.125 (14.56)	$p>0.050$	$p>0.050$
5×10^{-6}M	SLE 8.575 (8.15) C 8.275 (13.19)	$p>0.050$	$p>0.050$
1×10^{-5}M	SLE 10.350 (16.34) C 8.213 (12.75)	$p<0.010$	$p<0.010$
5×10^{-5}M	SLE 10.850 (14.28) C 8.588 (13.26)	$p<0.010$	$p<0.010$
1×10^{-4}M	SLE 11.050 (12.20) C 9.988 (12.67)	$p<0.010$	$p>0.050$
5×10^{-4}M	SLE 13.650 (25.36) C 12.400 (13.74)	$p<0.010$	$p>0.050$
1×10^{-3}M	SLE 16.240 (43.94) C 15.788 (24.55)	$p<0.010$	$p>0.050$

HYD(-) symbolises untreated lymphocytes; HYD(+) symbolises HYD-treated lymphocytes

Table 7.3. Frequencies of First, Second and Further Divisions (M1, M2, and ≧ M3, respectively) in HYD-Treated and Untreated Lymphocyte Cultures from a HYD Induced Lupus Patient (SLE) and His Controls (BF and Donor 1).
The data are base on observation of 50 randomly selected metaphases.

HYD Treatment (M)	PERCENT FREQUENCIES								
	SLE			BF			Donor 1		
	M1	M2	≧M3	M1	M2	≧M3	M1	M2	≧M3
0	6	30	64	22	46	32	18	30	52
1×10^{-6}	8	42	50	22	48	30	30	40	30
5×10^{-6}	8	48	44	18	54	28	30	46	24
1×10^{-5}	16	42	42	20	52	28	20	38	42
5×10^{-5}	26	42	32	24	42	34	34	42	24
1×10^{-4}	26	56	18	20	56	24	46	30	24
5×10^{-4}	44	52	4	50	44	6	50	50	0
1×10^{-3}	70	30	0	90	10	0	88	12	0

Discussion

The findings of this study indicate that lymphocytes from a hydralazine induced SLE patient are more sensitive to SCE induction by HYD than similarly treated control lymphocytes. The hypersensitivity of the cells from the patient was manifested by a significant increase in the SCE frequency at HYD concentrations which caused no increase in the SCE frequency of similarly treated control cell (Table 7.2.). However, at concentrations sufficient to increase the SCE frequencies of control lymphocytes, no difference between the SLE and the

control lymphocytes was noted (Table 7.2.). Unfortunately, no interpretable results could be obtained on the effects of HYD on cell cycle kinetics (Table 7.3.). The reasons for the observed differences in the response to HYD treatment remain unclear. This problem will remain intractable until hydralazine's mode of action has been fully elucidated.

The results presented in this study must be regarded with scepticism because only one HYD induced lupus individual was tested. It is unfortunate that better controls could not be obtained for this study and that it was impossible to get samples from other HYD induced lupus individuals. One can only point out that in this case a difference with respect to HYD induced SCEs was detected between the lupus patient's lymphocytes and his controls' lymphocytes. An extensive study will need to be performed in order to ascertain whether the difference picked up in this study is a true difference or merely a spurious result.

In conclusion, the observation that lymphocytes of a hydralazine sensitive individual are more susceptible to SCE induction by HYD raises the possibility that the SCE end-point might be used to identify individuals at risk of contracting HYD induced lupus. However, in the light of the results presented in Chapter 5, this suggestion must be regarded with caution since it was demonstrated there that inter-individual differences in response to chemical insult may be very wide-ranging.

CONCLUDING REMARKS

It is incontrovertible that the detection of SCE can only further our knowledge of cellular processes. Of course, it is also indisputable that the information gained from work on SCE would be greatly enhanced if the mechanism for its formation were fully understood. Because SCE has been used most frequently as an index of DNA damage, attempts have been made to correlate SCE induction with mutagenicity of established DNA damaging agents. Unfortunately, the correlation between SCE induction and the DNA damaging properties of the classical mutagen, ionizing radiation, is at best a poor one. With some chemical mutagens, however, there appears to be a linear relation between the induction of SCEs and mutations at the hypoxanthene-guanine phosphoribosyltransferase locus in CHO cells (Carrano et al., 1978). However, the ratio of induced SCEs to induced mutations is different for different chemicals (Carrano et al., 1978). This may be in part due to the fact that the agents used in the study of Carrano et al. (monofunctional alkylating agents: ethyl methanesulfonate, ethyl nitrosourea; bifunctional alkylating and crosslinking agent: mitomycin C; intercalating agent: proflavine) produce different lesions in DNA. In a paper recently accepted by Nature Evans and Vijayalaxmi have reported that the incidence of 8-azaguanine-resistant cells increases in a dose-dependent manner in lymphocytes exposed in vitro to mitomycin C and that this increase is paralleled by an increase in the SCE frequency in the same cell population. These authors suggest that SCEs and 8-azaguanine-resistance are both consequences or end-points of the interaction of MMC with DNA. However, they remark that 8-azaguanine resistance is not necessarily due to a mutation. As has been demonstrated in the experiments presented in Chapter 4, there is not a strict

relation between SCE induction and alkylation of the O-6 position of guanine which is thought to be one of the important lesions involved in mutagenesis. Therefore it is not surprising that comparisons between SCE induction and mutation induction are not as straight forward as one might hope they would be.

SCEs and chromosomal aberrations are not directly comparable end-points since agents such as X-rays and BLM induce vast quantities of aberrations but few SCEs. Reciprocally, agents such as UV light and EMS induce few aberrations relative to SCEs. However, the results presented in Chapter 3 indicate that some chromatid breaks are coincident with exchange and this suggests that in certain circumstances the processes of SCE and aberration formation may be interrelated. In view of this fact, it does not seem reasonable to uphold the often-voiced claim that SCE is a more sensitive assay for DNA damage than aberration induction (a claim which implies that SCE studies should be given precedence over aberration studies). Rather, it is important that both end-points be examined in parallel in order to gain some insight about the cellular processes which govern their induction.

It is unlikely that the SCE assay will be useful in monitoring exposure of individuals to low doses of radiation (Chapter 5). However, high SCE frequencies have been reported in patients treated with anti-tumor drugs (Perry and Evans, 1975, Nevstad, 1978, Raposa, 1978). Therefore SCE might be used as a probe for exposure of individuals to chemical mutagens although there are difficulties involved in determining what constitutes a "low" level of exposure and in deciding what cell populations to sample. Attempts have been made to use SCE as a marker for "cancer risk" (Cheng et al., 1979) and to monitor progression of patients with chronic myeloid leukemia (Kakati et al., 1978, Knuutila et al., 1978), acute myeloid leukemia (Knuutila et al., 1978), and acute lymphoblastic leukemia (Otter et al., 1979). The relation between changes in the SCE frequency and progression towards (or changes in) malignant states is at present un-

known, but from a clinical point of view it is worthwhile to devote some effort to research in this field.

Lymphocytes from a hydralazine-induced lupus individual may be more sensitive to *in vitro* SCE induction by hydralazine than similarly treated control lymphocytes. Of course, a sample size of one can hardly be regarded as adequate, but it would be of clinical importance to know whether the SCE assay can be used to identify individuals hypersensitive to hydralazine, a frequently used hypotensive drug.

Radio-sensitive lymphocytes of Down's syndrome individuals were not found to be hypersensitive to SCE induction by a radio-mimetic chemical, bleomycin (Chapter 5). However, the observation that large inter- and intra-individual differences exist in the proportion of first, second and further division cells in lymphocyte cultures treated with BLM raises the possibility that second division cell populations used for SCE scoring might not be strictly comparable between cultures.

In conclusion, SCE induction may be regarded as an index of interference with normal replicative processes. Identification of the lesions which cause such interferences and the mechanisms which deal with overcoming them should further our understanding of mutagenesis and perhaps of carcinogenesis.

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APPENDIX

Cavaglià, A.M.V. (1980) In vitro induction of sister chromatid exchanges in human peripheral lymphocytes by hydralazine, Mutation Research, 77, 383-385.